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Université de Paris-Sud

Centre d'ORSAY

THESE DE DOCTORAT D'ETAT ES SCIENCES

PATRICE L. MOREAU

Mécanisme des fonctions cellulaires induites chez Escherichia coli par les lésions de l'ADN. Principe d'un test pour la détection et l'analyse du mécanisme d'action des substances cancérogènes ou antitumorales.

Soutenue le 19 juin 1981

Jury : M. P. Vigier
Mme E. Moustacchi
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AVANT-PROPOS

Les connaissances que nous avons acquises au cours de la dernière décennie sur les mécanismes de réparation de l'ADN permettent d'envisager avec optimisme des progrès rapides et décisifs dans ce domaine. Il est devenu évident qu'outre les systèmes de réparation "constitutifs" déjà caractérisés, il devait exister des systèmes de réparation "induits" par les dommages de l'ADN. Un des systèmes de réparation inductibles, connu sous le nom de "réparation SOS" ou "réparation fautive induite",a suscité un grand intérêt puisqu'il semble représenter une voie majeure dans les processus de mutagenèse. Cet intérêt a été fortifié lorsque des mécanismes de "réparation fautive" ont été impliqués dans l'initiation de la cancérogenèse par les agents chimiques (Witkin, 1976).

Le développement des recherches sur les systèmes bactériens est à l'origine des progrès réalisés dans l'élucidation des mécanismes de réparation de l'ADN. Le système biologique le plus utilisé et le mieux connu, tant au niveau génétique que biochimique, est constitué par la souche bactérienne Escherichia coli K12 et ses phages, en particulier le phage tempéré lambda. Les modèles et méthodologies développés avec les systèmes bactériens sont aujourd'hui transposés, avec semble-t-il un certain succès, aux systèmes eucaryotes (Hanawalt et al., 1979, Radman, 1980).

On ne peut traiter de la réparation de l'ADN sans mentionner d'autres processus cellulaires induits par les lésions de l'ADN. Parmi ces processus, communément appelés "fonctions SOS", nous citerons en particulier l'induction de bactéries à produire des phages qui sont normalement maintenus à l'état dormant (induction lysogénique). Ce dernier processus a constamment servi de modèle pour expliquer le mécanisme d'induction des fonctions SOS (Devoret, 1981).

I N T R O D U C T I O N

MECANISMES DE REPARATION DE L'ADN CHEZ E. COLI

I. Réplication et réparation "fidèles" de l'ADN

Au cours de la croissance cellulaire, le maintien de l'information génétique contenue dans la molécule d'acide désoxyribonucléique (ADN) exige :

1. une réplication "fidèle" de l'ADN,
2. une réparation "correcte" des lésions qui apparaissent spontanément ou qui sont causées par des agents externes.

A. Fidélité de la réplication

La fidélité de la réplication est essentiellement maintenue grâce à deux types de contrôles.

1. Le premier opère pendant la réplication, et ce à trois niveaux successifs (Loeb et al., 1978). Les différences d'énergie de liaison entre les paires de bases correctement appariées selon le modèle Watson-Crick et celles incorrectement appariées font que le taux d'erreurs est de l'ordre de un pour cent au cours de la réplication (Mildvan, 1974). La discrémination entre les paires de bases correctes et incorrectes est augmentée d'un facteur 10 à 100 par un mécanisme de sélection des bases par les polymérases (Loeb, 1974). Enfin, à l'extrémité 3' du brin nouvellement synthétisé, il y a élimination des bases mal appariées par une activité exonucléolytique 3' → 5' associée aux polymérases (fonction "editing" ou "proofreading") (Brutlag & Kornberg, 1972).

2. Le second mécanisme de correction opère immédiatement après la synthèse d'un nouveau brin d'ADN. Il s'agit de l'élimination par une glycosylase spécifique (produit du gène ung) de l'uracile incorporée à la place de la thymine (Olivera et al., 1979) ou de l'excision, de façon plus générale, des bases mal appariées ("mismatch correction") (Wagner

& Meselson, 1976). Le brin d'ADN ayant servi de matrice pour la réplication serait spécifiquement reconnu par le fait que les résidus adénine, dans les séquences 5'G-A-T-C3', seraient méthylés, alors que le nouveau brin d'ADN ne serait pas encore totalement méthylé (Lacks & Greenberg, 1977). L'excision des nucléotides sur le brin nouvellement synthétisé se ferait essentiellement dans le sens 5' → 3' et pourrait s'étendre sur une longueur de 3000 nucléotides (Wagner & Meselson, 1976). Les produits de nombreux gènes (dam, mutH, mutL, mutS, uvrE) semblent être impliqués dans cette voie de réparation majeure (Glickman & Radman, 1980),mais seul le produit du gène dam a été caractérisé jusqu'alors (méthylase de la déoxyadénosine) (Marinus, 1976).

Les différents mécanismes de correction qui agissent pendant ou immédiatement après la réplication assurent à ce processus un haut degré de fidélité. On estime qu'il ne doit pas se produire plus d'une erreur pour 10^{10} nucléotides incorporés (Hartman, 1980).

B. Réparation pré-réplicative par excision des bases ou des nucléotides altérés

Si l'on met à part la réparation par photoréactivation, mécanisme de réparation propre aux dimères de pyrimidines qui exige une illumination des cellules (λ 310-400 nm) (Rupert, 1975 ; Hélène & Charlier, 1978), la réparation de l'ADN endommagé (spontanément ou par des agents externes) s'effectue essentiellement par un mécanisme d'excision des bases ou des nucléotides altérés (Laval, 1978).

Le mécanisme de réparation par excision des bases semble jouer un rôle plus important qu'on ne l'a cru tout d'abord (Lindahl, 1979). Il se produit selon le schéma général suivant :

1. Reconnaissance et excision des bases altérées par des N- glycosylases spécifiques conduisant à la production de sites apyrimidiques ou apuriniques (sites AP). Les lésions de l'ADN résultent d'évènements spontanés (déamination) ou sont produites par des agents alkylants. Les glycosylases caractérisées sont spécifiques de l'uracile (C → U) (produit du gène ung), de l'hypoxanthine (A → HX), ou de la 3-méthyle adénine (produit du gène tag) (Karran et al.,1980).

Dans le cas de sites apuriniques, une "insertase" pourrait insérer une base en remplacement de la base excisée, reconstituant ainsi directement l'intégrité de la molécule (Livneh et al.,1979).

2. De façon générale, il semble que les sites AP, produits spontanément ou à la suite de l'action de N-glycosylases, soient reconnus par des AP-endonucléases (en particulier par l'AP-endonucléase, produit du gène xthA). Les AP-endonucléases effectuent une incision dans la chaîne phosphodiester en 5' du site AP.

3. La dégradation de l'ADN se poursuit alors par l'activité d'exonucléases, notamment par l'activité exonucléolytique 5' → 3' associée à la polymérase I (polA). En fait, l'excision des nucléotides est apparemment couplée à la resynthèse de l'ADN. Les régions d'ADN ainsi réparées par excision-resynthèse ont une longueur totale de 20 à 30 nucléotides (Cooper & Hanawalt, 1972).

L'intervention des exonucléases V (recBC) et VII (xseA), des polymérases II (polB) et III (polC) peut provoquer une dégradation plus importante de l'ADN ("long patch"), ce qui nécessite alors la mise en oeuvre d'un nouveau système de réparation mutagène, la réparation SOS (Cooper & Hanawalt, 1972 ; Hanawalt et al., 1979) (voir chapitre suivant).

4. La continuité de la molécule d'ADN est finalement restaurée par l'activité d'une polynucléotide ligase (lig) (Olivera & Lehman, 1967 ; Youngs & Smith, 1977) qui effectue la jonction entre l'extrémité 3'OH du fragment resynthétisé et la molécule d'ADN.

Chez E. coli, les premières étapes de la réparation par excision sont généralement effectuées par les produits des gènes uvr A, B et C (Seeberg, 1978) qui ont un spectre d'action assez large puisqu'ils reconnaissent aussi bien les dimères de pyrimidines, induits par le rayonnement UV, que les lésions produites par la fixation covalente de produits chimiques sur l'ADN. Dans ce cas, il semble que l'excision de la lésion soit initiée par l'activité d'une endonucléase (l'endonucléase uvrAB) et non par un système glycosylase-AP endonucléase (Laval, 1978 ; Lehmann, 1980 ; Rothman, 1980).

C. Réparation post-réplicative

Le phénomène de réparation post-réplicative ("post-replication repair") a été décrit pour la première fois chez des bactéries uvr$^-$ exposées au rayonnement UV (Rupp & Howard-Flanders, 1968). Parmi les différents modèles proposés par Rupp et Howard-Flanders, celui qui est aujourd'hui généralement retenu suggère que la réplication de l'ADN s'interrompt momentanément en face de chaque dimère de pyrimidines pour être réamorcée, une fois cette lésion franchie, au prochain site de réinitiation d'un fragment d'Okazaki. Les "vides" ("gaps") laissés dans le fragment d'ADN naissant pourraient être réparés par un évènement de recombinaison entre le brin d'ADN nouvellement synthétisé et le brin parental de même polarité. Le matériel génétique ainsi échangé aurait une longueur au moins égale à 1000 nucléotides (Rupp et al.,1971) et pourrait contenir des dimères de pyrimidinesqui seraient ainsi progressivement retirés du brin parental irradié (Ganesan, 1974).

Les produits des gènes recA, recBC, uvrD, polA et polC ont été impliqués dans ce processus de réparation, mais les réactions intermédiaires qu'ils catalysent ne sont pas encore totalement élucidées (Hanawalt et al., 1979).

Il faut souligner que le dommage qui est réparé par la réparation post-réplicative n'est pas la lésion primaire de l'ADN mais la lésion qui est produite par la réplication. Il ne s'agit donc pas à proprement parler d'un système de réparation, mais plutôt d'un système qui permet de "tolérer" un certain nombre de "lésions résiduelles" dans un ADN qui se réplique.

D. Efficacité et fidélité des systèmes de réparation par excision et recombinaison

Si l'on estime l'efficacité des systèmes de réparation par le nombre de dimères de pyrimidines que peut "tolérer" une cellule, il est évident que le système de réparation par excision est de loin le plus efficace. Si une cellule E. coli de type sauvage peut tolérer l'induction de 3000 dimères dans son génome, une cellule mutante uvr$^-$, déficiente essentiellement dans le système de réparation par excision, ne peut plus en tolérer que 50 (Howard-Flanders, 1968).

Si les systèmes de réparation par excision et recombinaison sont efficaces, il est devenu évident que ces systèmes de réparation sont essentiellement fidèles ; ils ne peuvent rendre compte de l'apparition d'un nombre notable de mutations (Witkin, 1976).

II. Réparation post-réplicative "fautive"

A la suite des travaux de Rupp et Howard-Flanders, et pour rendre compte du fait que les UV n'induisent pas de mutations chez des bactéries portant la mutation exr (lexA), Witkin suggéra l'existence d'une seconde voie de réparation post-réplicative. Cette réparation serait responsable de la

mutagenèse induite par les UV. Elle opérerait en comblant les vides laissés dans l'ADN, non pas par un évènement de recombinaison, mais par une synthèse qui pourrait s'effectuer en face des dimères de pyrimidines. Cette synthèse s'accompagnerait évidemment d'une forte probabilité d'erreurs (Witkin, 1967).

En 1971, Defais et al. émirent l'hypothèse selon laquelle le système de réparation mutagène suggéré par Witkin serait induit par les lésions résiduelles de l'ADN contrairement aux systèmes de réparation par excision et recombinaison qui seraient essentiellement constitutifs. Cette idée sera développée par Radman sous le nom de "réparation infidèle induite" ("inducible error-prone repair) ou "réparation SOS" (Radman, 1975).

L'hypothèse de la réparation SOS repose en particulier sur l'observation que la mutagenèse présente de nombreuses analogies avec un autre processus cellulaire : l'induction de bactéries lysogènes à produire des phages. Ces deux processus sont en effet induits par les mêmes doses d'irradiation UV, ainsi que par les mêmes agents qui, de façon symptomatique, sont également connus pour être des cancérogènes majeurs (Lwoff, 1953). Enfin, l'induction de ces deux phénomènes requiert l'expression des mêmes gènes recA et lexA, ainsi qu'une synthèse protéique de novo.

Une autre observation qui est à l'origine de l'hypothèse de la réparation SOS est celle faite par Weigle en 1953 : si des bactéries sont exposées à de faibles doses d'irradiation UV avant d'être infectées par des phages λ eux-mêmes fortement irradiés, la survie et la mutagenèse des phages sont augmentées. Les phages sont dits "réactivés". Devoret et al. (1975) ont montré que la réactivation de phages irradiés, ou Weigle-réactivation, est due à un processus de réparation nouveau, différent des systèmes de réparation par excision ou recombinaison.

Radman (1975) et Witkin (1976) ont suggéré que ce nouveau système de réparation inductible devait être responsable de la mutagénèse bactérienne.

III. Hypothèse des fonctions SOS

Dès 1967, Witkin avait émis l'hypothèse selon laquelle certaines fonctions cellulaires, notamment celles qui sont responsables de la synthèse d'un inhibiteur de la division cellulaire, seraient sous le contrôle de répresseurs cellulaires. Ces répresseurs seraient inactivés par un "inducteur universel" produit quand la synthèse de l'ADN est perturbée. En 1976, Witkin étendit ce concept de dérépression génique à toutes les fonctions cellulaires, ou "fonctions SOS", induites en réponse à l'endommagement de l'ADN et dépendant de l'expression des gènes recA et lexA.

Les fonctions SOS regroupent un certain nombre de processus cellulaires qui ont pour but d'augmenter la survie d'une cellule irradiée (ou de ses phages quand celle-là est lysogène). Ces fonctions comprennent notamment :

- l'activité de réparation SOS,
- la synthèse de l'inhibiteur de la division cellulaire,
- la réinitiation "aberrante" de la synthèse de l'ADN à l'origine de réplication,
- la synthèse de l'inhibiteur de l'activité exonucléase V,
- la synthèse de la protéine X,
- l'induction du prophage λ dans des bactéries lysogènes.

Au cours des cinq dernières années, l'hypothèse unitaire des "fonctions SOS" a eu l'immense mérite de susciter de très nombreux travaux grâce auxquels notre compréhension des mécanismes de réparation de l'ADN a fait d'immenses progrès.

IV. Mécanisme des fonctions SOS

En 1976, McEntee et coll. ont caractérisé le produit du gène recA grâce à l'isolement d'un phage transducteur λrecA$^+$: la protéine RecA est composée de quatre sous-unités

identiques de 39000 daltons connues jusqu'alors sous le nom de protéine X (Inouye & Pardee, 1970; McEntee, 1977; Gudas & Mount, 1977).

Roberts et coll. ont alors établi que la protéine RecA clive le répresseur du prophage λ et déclenche ainsi l'induction de bactéries lysogènes (Roberts et al., 1978; Craig & Roberts, 1980).

Le fait que la synthèse de la protéine RecA augmente de 5 à 10 fois dans des cellules induites (Gudas & Pardee, 1976) a conduit à penser que l'amplification de la protéine RecA pouvait être responsable de l'expression des différentes fonctions SOS (McEntee, 1977; Roberts et al. 1978; Satta et al. 1979).

Quel serait le mécanisme d'induction de la synthèse de la protéine RecA ? Deux types d'hypothèses prévalaient :

1) Un complexe comprenant les protéines LexA et RecA contrôlerait l'expression du gène recA (Moody et al., 1973). L'induction du gène recA résulterait soit d'une compétition pour le complexe répresseur entre le site opérateur du gène recA et les lésions de l'ADN (Sussman & Ben Zeev, 1975), soit d'une dissociation du complexe répresseur résultant d'une interaction avec des produits de dégradation de l'ADN endommagé (Bailone et al., 1979).

Ces hypothèses étaient basées en particulier sur le fait que l'antipaïn, un inhibiteur de protéase, semblait empêcher le clivage du répresseur du prophage λ sans pour autant bloquer l'induction de la synthèse de la protéine RecA (Sedgwick et al., 1978).

2) La protéine LexA, répresseur du gène recA, serait détruite par la protéine RecA activée par des cofacteurs tels que des produits de dégradation de l'ADN (McEntee, 1977; Gudas & Mount, 1977).

Des résultats récents ont clairement établi que la dernière hypothèse était la bonne :

1) La protéine LexA est le répresseur du gène recA (Little & Harper, 1979) ainsi que celui de nombreux autres "gènes SOS" parmi lesquels se trouvent le gène lexA lui-même et le gène umuC qui contrôle la réparation mutagène (Kenyon & Walker, 1980; Quillardet et al., 1981). In vitro, la protéine LexA bloque la transcription des gènes recA et lexA (Brent & Ptashne, 1981).

2) La protéine LexA est clivée par la protéine RecA, in vitro, au cours d'une réaction analogue à celle décrite pour le clivage du répresseur du prophage λ (Little et al., 1980). Horii et al.(1981) ont montré que la protéine LexA contient une région similaire à celle où se produit le clivage du répresseur du prophage λ.

3) Dans des bactéries lysogènes, le clivage du répresseur du prophage λ n'exige pas une amplification de la protéine RecA (Moreau et al.,1980a; Devoret et al.,1981).

Ces résultats démontrent à l'évidence que la conversion de la protéine RecA en protéase hautement spécifique constitue le véritable évènement inducteur des fonctions SOS.

Si la protéine RecA a une activité protéolytique essentielle pour l'induction des fonctions SOS, elle joue également un rôle fondamental dans la survie des cellules en tant que protéine de "structure" (Quillardet et al., 1981). En ce collant en longs filaments sur les régions d'ADN en simple brin (West et al., 1980), la protéine RecA a une double fonction :

1) Elle prévient la dégradation par les exonucléases cellulaires de l'ADN endommagé (Gudas & Pardee, 1976).

2) Elle permet la recombinaison génétique. En fait, la protéine RecA exprime alors une seconde activité enzymatique: une activité ATPasique (Ogawa et al., 1978). La protéine RecA

ne peut se fixer à l'ADN que si elle est couplée à un nucléoside triphosphate (ATP ou dATP). Après appariement des brins d'ADN homologues (Cunningham et al., 1981), la protéine RecA induirait sa propre chasse du complexe de recombinaison en hydrolysant l'ATP (McEntee et al., 1979).

V. Implications de l'hypothèse des fonctions SOS pour la cancérogenèse chimique.

L'hypothèse selon laquelle des lésions de l'ADN induisent l'expression de nombreux phénomènes aussi bien génétiques (mutagenèse) qu'épigénétiques (induction de provirus) (Toman et al., 1980) permet de concilier un certain nombre de modèles, apparemment contradictoires, concernant l'induction de la cancérogenèse par les produits chimiques (Miller, 1978; Echols, 1981).

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L'INDUCTEST :

UN TEST BACTERIEN POUR LA DETECTION DES CANCEROGENES POTENTIELS

La plupart des agents cancérogènes ont une activité mutagène dans des systèmes bactériens. Cela implique-t-il que l'induction de la cancérogénèse soit due à une accumulation de mutations ? Dès 1953, Lwoff avait pressenti l'existence de relations causales plus complexes en notant que de nombreux agents mutagènes et cancérogènes induisent également le développement végétatif de virus (prophages) qui sont normalement maintenus à l'état dormant dans des bactéries dites lysogènes. Or, l'induction de tels prophages semble résulter de l'inactivation par clivage protéolytique des répresseurs qui empêchent normalement leur développement.

Si la relation entre activité cancérogène et activité inductrice de prophages dans des bactéries lysogènes devait se révéler aussi bonne que la relation entre activité cancérogène et activité mutagène, cela aurait des implications importantes pour la compréhension du mécanisme d'induction de la cancérogénèse par les agents chimiques. Par analogie avec les systèmes bactériens, l'initiation de la cancérogénèse pourrait alors s'expliquer à travers l'induction par les lésions résiduelles de l'ADN de nouvelles fonctions cellulaires, ou fonctions SOS : l'événement inducteur de la cancérogénèse serait alors vu comme la dérépression de gènes, et non comme la simple accumulation de mutations.

Pour tester ces hypothèses, nous avons entrepris de développer un nouveau test d'induction lysogénique qui permette de comparer dans les mêmes souches bactériennes l'activité inductrice du prophage λ et l'activité mutagène d'un produit.

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Microbiology

Prophage λ induction in *Escherichia coli* K12 *envA uvrB:* A highly sensitive test for potential carcinogens

(aflatoxin B1/benzo[*a*]pyrene/7,12-dimethylbenz[*a*]anthracene)

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ABSTRACT **A simple, inexpensive, and sensitive test for potential carcinogens based upon the property of carcinogens to induce prophage λ is described. By using chemicals activated with microsomal enzymes and *E. coli* K12 permeable (*envA*) tester bacteria also deficient in DNA repair (*uvrB*), the range of carcinogens detected in a lysogenic induction test (inductest) has been extended. We have provided the evidence that, after activation, carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induce prophage λ. Three variants of the test have been developed (inductests I, II, and III), which are as sensitive as the mutagenicity test of Ames *et al.* [Ames, B. N., McCann, J. & Yamasaki, E. (1975) *Mutat. Res.* 31, 347–364]. Inductests II and III provide a quantitative estimation of the inducing activity of a carcinogen. With the latter test, one can determine: (*i*) the cellular toxic effect of a carcinogen and (*ii*) the kinetics of appearance and disappearance of active metabolites. For two series of chemicals, aflatoxins and benz[*a*]anthracenes, there is a good correlation between their carcinogenic activity in rodents and their prophage inducing activity in bacteria. The fact that the majority of the cell population is induced makes it possible to test the inducing activity of carcinogens at the biochemical level, e.g., by measuring λ repressor inactivation.**

More than a hundred carcinogens have recently been shown to be mutagens in *Salmonella typhimurium* (1, 2). The correlation between carcinogenicity and mutagenicity provides a useful test for potential carcinogens.

Recent work suggests that when *Escherichia coli* K12 bacteria are treated with ultraviolet light (3–6) or aflatoxin B1 (a potent carcinogen) (7, 8), persisting chromosomal DNA lesions induce a metabolic pathway that leads to mutagenesis as well as to the induction of prophage λ. One expects that carcinogens should induce prophage λ as well as they induce mutagenesis.

Until now, only a limited number of carcinogens have been shown to induce prophage λ in *E. coli* K12 (9–11). This relative failure can be ascribed to the lack of a proper technique. Most chemicals cannot penetrate bacteria (12), and, to be active, carcinogenic compounds have to be metabolized by mammalian liver enzymes (1, 2, 13).

We describe here an improved test for potential carcinogens based upon lysogenic induction. The procedure involves the use of activated chemicals and new permeable tester bacteria (*envA*) also deficient in DNA repair (*uvrB*). Three variants of this efficient, simple, and inexpensive lysogenic induction test (inductest) are reported and discussed.

MATERIALS AND METHODS

Bacterial Strains. All strains were derived from D21 *env*$^+$ and D22 *envA* (14) except GY 4015, an Amp^R derivative of C600. A pedigree of the strains used will be sent upon request.

Abbreviation: Me_2SO, dimethylsulfoxide.

Media and Culture. LB: Difco yeast extract, 5 g; Difco tryptone, 10 g; NaCl, 10 g; water, 1 liter. LBE: LB supplemented with 0.2% glucose and 20% medium E of Vogel and Bonner. LA-Ca: LB, 1 liter supplemented with 0.2% glucose and 2.5 mM $CaCl_2$; Biomar agar, 15 g. GT-amp: peptone, 8 g; Difco tryptone, 5 g; NaCl, 5 g; Biomar agar, 12 g; water, 1 liter; supplemented with 10 μg/ml of D-ampicillin. Soft agar: Difco agar, 7.5 g; water, 1 liter.

In all experiments, λ lysogens were grown as follows: an overnight culture in LBE medium at 37° was diluted 1:100 into fresh LBE medium and incubated with aeration at 37° up to about 4×10^7 viable cells per ml ($OD_{650} = 0.3$).

Chemicals. NADP and glucose-6-phosphate were from Sigma; aflatoxin B1, aflatoxin G1, and sterigmatocystin from Makor; and benzo[*a*]pyrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and 3-methylcholanthrene from Fluka. Benzo[*a*]pyrene 4,5-oxide was kindly provided by D. M. Jerina.

Carcinogens were dissolved and diluted when needed in dimethylsulfoxide (Me_2SO), spectrophotometric grade (Merck), and kept in the dark.

Metabolization of Carcinogens. Except where otherwise stated, the metabolizing mixture consisted of 1 volume of postmitochondrial fraction (13) and 6 volumes of NADPH-generating system: 150 mM phosphate buffer (pH 7.5), 3 mM NADP, 10 mM glucose-6-phosphate, and 12 mM $MgCl_2$. The latter was sterilized by filtration and stored at 4°.

The postmitochondrial fraction was from rats that had received either three daily injections of 75 mg/kg of phenobarbital sodium or two daily injections of 20 and 10 mg/kg of 3-methylcholanthrene in the days preceding their being killed.

Viability of *envA* (λ) Lysogens in Liquid and on Solid Media. Starting from a saturated culture, growth of *envA* bacteria is exponential for a few generations. The exponential growth phase of *envA* (λ) lysogens cannot be maintained by repeated dilutions into fresh rich medium since bacteria begin to die. A similar situation is found when *envA* bacteria are plated because colony formation involves a long exponential growth phase (14). We found that *envA* lysogens remain alive when plated with *E. coli* bacteria killed with streptomycin. Practically, 0.1-ml dilutions of *envA* bacteria were plated with 3 ml of soft agar and 0.3 ml of strain GY 4015 at about 2×10^9 cells per ml onto LA-Ca plates containing 200 μg/ml of streptomycin. Under these conditions, *envA* bacteria have an efficiency of colony formation 5-fold less than *env*$^+$ cells taken at the same optical density. This result is consistent with the fact that *envA* bacteria have on average 5 cell units per colony former (15).

We hypothesize that the streptomycin-killed bacteria provide a factor essential for the exponential growth of *envA* bacteria; the factor seems to be accumulated in the resting cells. This factor of viability might be either an enzyme or an activator

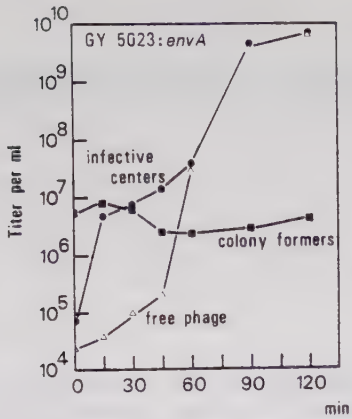

FIG. 1. One-step growth curve of phage λ induced by aflatoxin B1 metabolites. To 1.4 ml of metabolizing mixture were added a sample of 0.3 ml of *envA* (λ) bacteria at 4×10^7 cells per ml and then 40 μl of Me_2SO containing 8.7 μg of aflatoxin B1 (final concentration 5 μg/ml). The preparation was incubated with aeration in the dark at 37°. At times indicated on the abscissa, diluted samples in 10 mM $MgSO_4$ were assayed for colony formers (■), infective centers (●), and free phage (△). Microsomes were from rats treated with phenobarbital.

of the enzyme, *N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) found to be reduced in *envA* bacteria (16). Our finding should help to elucidate the physiology of the *envA* defect.

Kinetics of Lysogenic Induction. A λ lysogen, when induced, gives rise to an infective center which bursts and liberates free phage. Free phage are resistant to chloroform, unlike infective centers (17); free phage and infective centers are both detected as plaques on a lawn of phage λ indicator bacteria. An example of a one-step growth curve of phage λ induced by carcinogenic metabolites, e.g., aflatoxin B1 metabolites, is shown in Fig. 1. When *envA* (λ) bacteria were mixed with aflatoxin B1 and the metabolizing mixture, prophage λ was readily induced; the lysogens began to lyse between 30 and 40 min at 37°. Before 30 min of incubation, the amount of free phage was negligible; plaques were only produced by infective centers. The number of the infective centers formed was not equal to the decrease in bacterial survivors; this can be accounted for by the fact that *envA* bacteria form chains of about 4 to 5 cell units (15). A single induced lysogen per chain is enough to give rise to an infective center, whereas the loss of a colony occurs only if all cells of a chain are killed.

Efficiency of Lysogenic Induction is calculated as the ratio of the number of infective centers to the number of original viable lysogens. To measure infective centers, the treated λ lysogens were plated onto GT-amp plates with 3 ml (or 2.5 ml in inductests I and II) of soft agar containing 0.3 ml of indicator bacteria GY 4015 at about 2×10^9 cells per ml. Ampicillin prevents the noninduced bacteria from multiplying and, therefore, from releasing phage during the overnight incubation of plates. This antibiotic eliminates spontaneous phage production but does not inhibit the induced lysogens to form infective centers (18). This technique was applied to *envA* (λ) lysogens since they are phenotypically ampicillin-sensitive (14).

In inductest III, to estimate the efficiency of lysogenic induction of any carcinogen, incubation in liquid at 37° was never carried out any longer than 25 min in order to avoid the burst of induced lysogens. However, in the case of a weak λ inducer, it is possible to magnify the number of the detected plaques by allowing the burst of the induced lysogens: then, free phage instead of infective centers are measured.

RESULTS

The Inductests. When an inductest is performed the following are used: (*a*) the tester bacteria, such as *envA uvrB* (λ) lysogens; (*b*) the λ indicator Amp^R bacteria to reveal the phage produced by induction; (*c*) the drug to be tested dissolved in Me_2SO; (*d*) if required, the metabolizing mixture that transforms the drug into active products; and (*e*) GT-amp plates.

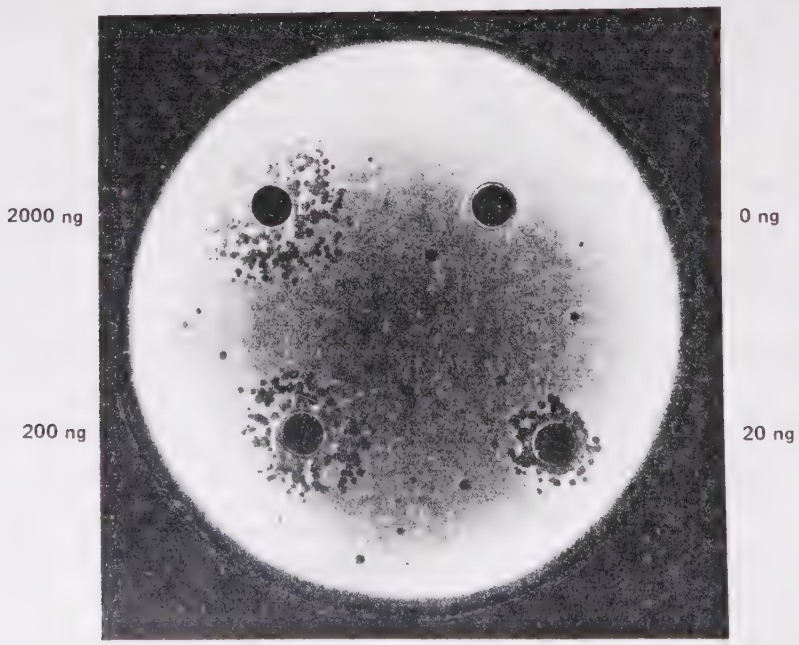

FIG. 2. Spot test (inductest I) for prophage λ induction by aflatoxin B1 metabolites. The following were poured on a GT-amp plate: 2.5 ml of soft agar with 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml and 1 ml of *envA uvrB* (λ) bacteria at about 8×10^3 cells per ml diluted 1:8 in the metabolizing mixture. Four filter paper discs were placed on the agar when it had hardened. On each filter were deposited 20 μl of Me_2SO containing one of the four following concentrations of aflatoxin B1: 0, 20, 200, or 2000 ng. The plate was incubated overnight at 37°. Microsomes were from rats treated with phenobarbital.

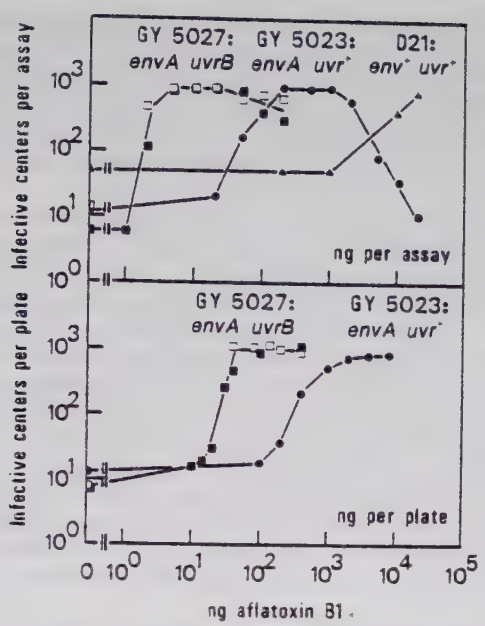

FIG. 3. Detection of aflatoxin B1: influence of *envA* and *uvrB* mutations. (Upper panel) Inductest III. One volume of bacteria of genotype indicated on the graph at about 2×10^4 cells per ml was mixed with 7 volumes of metabolizing mixture; 200-μl aliquots were distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Each preparation was gently agitated at 37° in the dark for 15 min and then poured onto GT-amp plates with 3 ml of soft agar containing 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. Prophage λ induction was measured as infective centers formed after overnight incubation at 37°. Microsomes were from rats treated with phenobarbital. (Lower panel): Inductest II. A sample of bacteria at about 4×10^3 cells per ml and the metabolizing mixture were mixed as described above; 1-ml aliquots were distributed into test tubes containing 20 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Preparations were then poured onto GT-amp plates with 2.5 ml of soft agar and with 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. Prophage λ induction was measured as infective centers formed after overnight incubation at 37°. Microsomes were from rats treated with phenobarbital.

Three variants of the test have been designed that differ with respect to how and when *a*, *b*, *c*, and *d* are mixed.

Inductest I: A qualitative spot test. The carcinogen is spotted onto plates directly or onto paper filters; the soft agar contains the λ lysogens, the phage λ indicator bacteria, and the metabolizing mixture. As soon as metabolization of the drug occurs, metabolites diffuse in the soft agar and penetrate the λ lysogens, which are induced: plaques appear around the spots after overnight incubation at 37° (Fig. 2). The drug diffusing out of a spot forms concentric rings of decreasing concentrations.

Inductest I is a simple procedure for the screening of a large number of chemicals. This test can also easily assess whether, in order to be active, a compound must be metabolized or not: aflatoxin B1 (20 ng) (Fig. 2) and 7,12-dimethylbenz[*a*]anthracene (4 μg) were found to be active only when metabolized, whereas benzo[*a*]pyrene 4,5-oxide (1 μg) and mitomycin C (0.1 μg) were active *per se*.

Inductest II: A quantitative test on plate. Five hundred to 1000 lysogenic cells are mixed with a definite amount of a carcinogen, of the metabolizing mixture, and of the phage λ indicator bacteria, and poured onto GT-amp plates.

Fig. 3 (lower panel) and Fig. 4 show that, at optimal input drug concentrations, all the treated cells were induced except when 3-methylcholanthrene was used. This compound seems highly toxic. The induction curves as a function of the drug concentration are approximately two-hit.

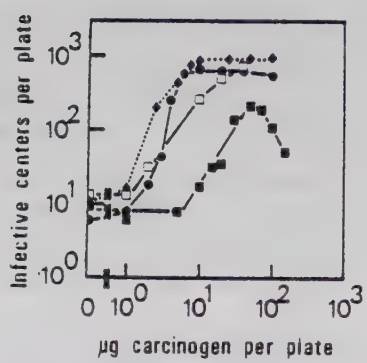

FIG. 4. Detection of polycyclic hydrocarbons in inductest II. GY 5027: *envA uvrB* (λ) bacteria were treated as in the legend of Fig. 3 (lower panel) with increasing amounts (indicated on the abscissa) of 7,12-dimethylbenz[*a*]anthracene (♦), benzo[*a*]pyrene (●), benzo[*a*]pyrene 4,5-oxide (□), or 3-methylcholanthrene (■). In the case of benzo[*a*]pyrene 4,5-oxide, the metabolizing mixture was omitted. Microsomes were from rats treated with phenobarbital, or with 3-methylcholanthrene in the case of benzo[*a*]pyrene.

Inductest III: A quantitative test in liquid medium. A definite number of lysogens (500–1000) and a given amount of a carcinogen and of the metabolizing mixture are mixed and incubated at 37°. Metabolization of the drug and cell penetration of the metabolites are stopped by dilution in soft agar and plating.

The sensitivity of inductest III is generally higher than that of inductest II (Fig. 3). In the case of water-insoluble compounds, however, only about 50% of the treated lysogens were induced in liquid instead of 100% in the soft agar (Figs. 4 and 5). Diffusion of the water-insoluble metabolites and their penetration into lysogens is slow; incubation for 15 or 25 min in liquid was not enough to bring about full induction of the lysogens.

To estimate the kinetics of appearance and disappearance of the carcinogenic metabolites, we first incubated the metabolizing mixture at 37° with the carcinogen and, at various times, added the lysogens and incubated for 15 min; the frequency of infective centers produced was taken as an estimate

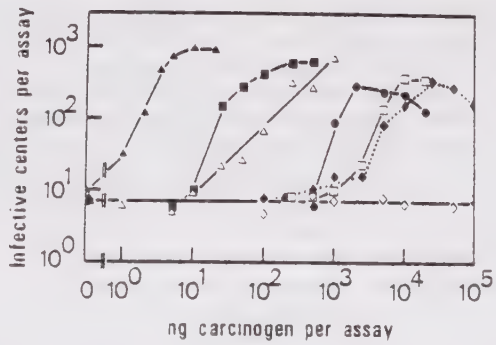

FIG. 5. Comparison of λ inducing activity of various carcinogens (inductest III). GY 5027: *envA uvrB* (λ) bacteria were treated as in the legend of Fig. 3 (upper panel) with increasing amounts (indicated on the abscissa) of aflatoxin B1 (▲), aflatoxin G1 (■), mitomycin C (△), benzo[*a*]pyrene (●), benzo[*a*]pyrene 4,5-oxide (□), 7,12-dimethylbenz[*a*]anthracene (♦), or benz[*a*]anthracene (◇). In the case of mitomycin C and benzo[*a*]pyrene 4,5-oxide, the metabolizing mixture was omitted. Treatment with 7,12-dimethylbenz[*a*]anthracene, benz[*a*]anthracene, and benzo[*a*]pyrene 4,5-oxide was for 25 min. Microsomes were from rats treated with phenobarbital, or with 3-methylcholanthrene in the case of benzo[*a*]pyrene.

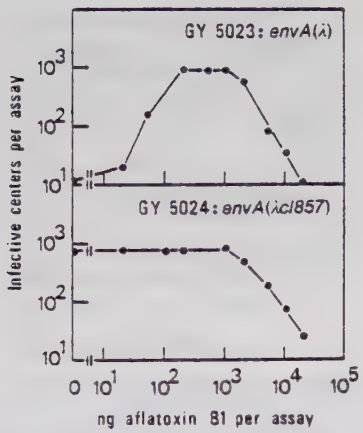

FIG. 6. Capacity of cells treated with aflatoxin B1 to reproduce prophage λ. (Lower panel) One volume of *envA* (λ*cI857*) bacteria at about 2×10^4 cells per ml was mixed at 32° with 7 volumes of metabolizing mixture; 200-μl aliquots were distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of aflatoxin B1 (indicated on the abscissa). Each preparation was gently agitated at 32° in the dark for 25 min and then poured onto GT-amp plates with 3 ml of soft agar containing 0.3 ml of GY 4015 indicator bacteria at 2×10^9 per ml. The development of the induced λ prophage was measured as infective centers formed after overnight incubation at 42°. Microsomes were from rats treated with phenobarbital. (Upper panel) (Inductest III) *envA* (λ) bacteria were treated as described above except that incubation was for 15 min at 37°. Infective centers on the ordinate were measured after overnight incubation at 37°.

of the level of active metabolites. We found that the level of the active metabolites of aflatoxin B1 decreased immediately almost exponentially as a function of the time of preincubation; the half-life of aflatoxin B1 metabolites was about 10 min at 37°.

Sensitive Tester Lysogens. Fig. 3 (upper panel) shows that the combination of *envA* and *uvrB* mutations increases the detection of aflatoxin B1 metabolites more than 4000 times. The *envA* mutation augments the sensitivity of the induction test 100-fold. The fact that the *uvrB* mutation also increases the cell sensitivity to the metabolites of aflatoxin B1 and benzo[*a*]pyrene (data not shown) indicates that these metabolites produce bacterial DNA lesions which are removed by the excision repair pathway.

Drug Metabolization. We have routinely tested potential carcinogens using microsomes from rats treated with phenobarbital, except in the case of benzo[*a*]pyrene, in which microsomes from rats treated with 3-methylcholanthrene were used (19). With sterigmatocystin both types of microsomes gave identical results. With aflatoxin B_1, however, although the efficiency of induction was unchanged, the capacity to reproduce the phage decreased when the microsomes used were from rats treated with phenobarbital. This is in agreement with the previous observations that more toxic metabolites are produced by microsomes from phenobarbital-treated rats (13).

Toxicity Test for Chemical Carcinogens. All the known inducers of prophage λ have two antagonistic effects: (*i*) they trigger the multiplication of the prophage and (*ii*) they cause damage to the prophage as well as to the host bacterium so that the cell's capacity to reproduce the induced prophage is affected. At high ultraviolet doses, lysogenic induction occurs but may go unnoticed by lack of production of mature phage particles; it may be so for any chemical carcinogen when used at high concentration.

By replacing λ lysogens with λ*cI857* lysogens in inductest III, we established a means of testing the range of toxicity of a chemical compound. A culture of λcI857 lysogens is submitted at 32° to the tested drug; at this temperature the prophage is not induced (*ind*$^-$). The culture is shifted to 42°; all cells are then induced because, at this temperature, the λ*cI857* mutant repressor is inactivated. Any reduction in the number of infective centers will be due to the deleterious effect of the drug on the cell capacity to reproduce the prophage.

On Fig. 6 (lower panel) we see that at low concentrations of aflatoxin B1, 100% of the λ*cI857* treated cells are induced; at higher doses, the descending part of the curve of the λ*cI857* infective centers parallels that of the λ lysogens (Fig. 6, upper panel); this shows the toxic effect of the metabolites.

Carcinogenic Activity and Prophage λ Induction. Sterigmatocystin is, as is aflatoxin B1, a widely disseminated mycotoxin and a powerful carcinogen (20). We have found that, when metabolized, these two related compounds induced prophage λ at similar low concentrations (data not shown). Aflatoxin G1 induces tumors in the rat with a considerably reduced potency as compared to that of aflatoxin B1 (20). Similarly, the input amount of aflatoxin G1 needed to induce all the treated lysogens was higher than that of aflatoxin B1 (Fig. 5).

Polycyclic hydrocarbons with a strong carcinogenic activity, such as benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, and 3-methylcholanthrene, induced prophage λ when metabolized (Figs. 4 and 5). In contrast, benz[*a*]anthracene, which has a questionable carcinogenic activity, failed to induce prophage λ in inductest II or III. Benzo[*a*]pyrene 4,5-oxide is the most stable of the benzo[*a*]pyrene epoxide metabolites (21). We have found that, as λ inducer, it was active *per se*, but less efficient than metabolized benzo[*a*]pyrene.

DISCUSSION AND CONCLUSIONS

Until now, lysogenic induction used as a test for chemical carcinogens had been of limited value for two main reasons: bacteria are not permeable to most chemical compounds (12) and, even if they are, many of the chemicals have to be metabolized to become active carcinogens (22). We have extended the range of carcinogens detected as prophage λ inducers by using permeable mutant lysogens and rat liver homogenates which duplicate mammalian metabolism. Apparently not altered in the lipopolysaccharide carbohydrate cell wall layers, *envA* bacteria are more permeable than *lps* mutants to compounds like gentian violet. The rate of gentian violet uptake by *envA* bacteria is similar to that of spheroplasts made by lysozyme-EDTA treatment (12). It seems likely that the *envA* mutation causes as much permeation as the bacterial cell envelope can tolerate.

The three inductests we developed are as sensitive as the mutagenicity test of Ames *et al.* (1). Moreover, inductests II and III provide a quantitative estimation of the λ inducing activity of a carcinogen. Inductest III possesses unique advantages: (*i*) the cellular toxic effect of carcinogens can be easily estimated by using λ*cI857* instead of λ lysogens, (*ii*) the kinetics of appearance and disappearance of active metabolites can be determined since drug metabolization and cell penetration of metabolites can be stopped at will by dilution and plating, and (*iii*) smaller quantities of the metabolizing mixture are needed and smaller amounts of the tested drug can be used (mainly in the case of water-soluble compounds).

We have provided the evidence that, after activation, carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene induce prophage λ. Furthermore, for two series of chemicals, we found that there is a good correlation between their carcinogenic activity in rodents

and their prophage inducing activity in bacteria. Aflatoxin B1 is a potent carcinogen as well as a potent λ inducer, whereas aflatoxin G1 is weak in both cases. Benz[*a*]anthracene, a questionable carcinogen, does not induce prophage λ even though it is a potent mutagen (2); its carcinogenic derivative, 7,12-dimethylbenz[*a*]anthracene is a λ inducer as well as a mutagen.

The use of lysogenic induction to study the cellular action of carcinogens has some advantages, one of which being the fact that, since the majority of the cell population is induced, one can test the inducing activity of carcinogens at the biochemical level, e.g., by measuring λ repressor inactivation.

The results of mutagenicity and lysogenic induction tests show that there is a corroboration of carcinogenic activity in eukaryotes with the ability of the very same chemicals either to induce a prophage or to produce mutations in prokaryotes. Such a correlation leads to postulate that: (*i*) the active forms of carcinogens *in vivo* are like those generated *in vitro* by mammalian liver enzymes, and (*ii*) DNA lesions induced in eukaryotes by carcinogens are similar to those induced in prokaryotes. Recent evidence suggests that, in bacteria treated with ultraviolet light (3–6) or aflatoxin B1 metabolites (7, 8), the DNA lesions, which persist after pyrimidine dimer excision repair, induce a metabolic pathway leading to mutagenesis, lysogenic induction, and cell filamentation. We hypothesize that, in eukaryotes, there exists a similar pathway which leads to carcinogenesis (8). With the aforementioned considerations in mind, one would surmise that if a chemical responds positively to the test of mutagenesis (mutatest) and to the test of lysogenic induction (inductest), it will cause cancer.

Unpublished experiments of M. Yarmolinsky to set up an inductest type I stimulated us in developing the inductests. We thank him for his help in the preparation of the manuscript. We are thankful to Y. Moulé and A. Sarasin for providing rat liver microsomal fractions as well as instructing us in their preparation. We are also grateful to many of our colleagues for the gift of various carcinogens. P.M. received a fellowship from Ligue Nationale Française contre le Cancer. Grants from this organization and Fondation pour la Recherche Médicale are gratefully acknowledged.

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Potential Carcinogens Tested by Induction and Mutagenesis of Prophage λ in *Escherichia coli* K12

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More than a hundred carcinogens have been shown to be mutagens in *Salmonella typhimurium* (McCann et al. 1975; Sugimura et al. 1976). The correlation found between carcinogenicity and mutagenicity in prokaryotes has led Ames (1976) to suggest that if a chemical is a mutagen for *Salmonella,* it is likely to have a carcinogenic activity. The factual correlation between carcinogenicity and mutagenicity in prokaryotes implies that there must exist a basic biological process common to prokaryotes and mammalian cells. This has led to the hypothesis that cell transformation results from the accumulation of mutations in the chromosomal DNA (McCann and Ames 1976; Cairns 1975 and this volume). In this paper, we discuss this notion and present a test for potential carcinogens based upon lysogenic induction as already proposed by Lwoff (1953). Lysogenic induction is a process whereby a virus in the dormant state (prophage) develops into a virus (phage) (Lwoff et al. 1950; Borek and Ryan 1973).

Some recent work in our laboratory (Goze et al. 1975; Sarasin et al. 1976; Devoret et al. 1976) has shown that many biological phenomena are expressed when *Escherichia coli* bacteria are treated with aflatoxin B_1, a potent carcinogen: (1) mutagenesis and repair of an ultraviolet (UV)-damaged phage λ infecting them, (2) induction of prophage λ when the bacteria are lysogenic for it, and (3) cell filamentation. These data have been interpreted by postulating that chromosomal DNA lesions persisting after constitutive repair induce a complex cellular metabolic pathway, the *SOS pathway* (see Discussion), which results not only in mutagenesis but also in the occurrence of the phenomena enumerated above. For all these experimental and theoretical reasons, one would expect carcinogens to be as good lysogenic inducers as they are mutagens.

In the past, only a limited number of carcinogens have been shown to induce lysogenic bacteria (Lwoff 1953; Geissler 1967; Heinemann 1971). This relative failure was due to the lack of a proper technique. In a previous publication (Moreau et al. 1976), we described an improved test for poten-

tial carcinogens based upon the induction of prophage λ in *E. coli* (inductest). Three variants of this efficient, simple, and inexpensive test are evaluated here as to their utilization and significance.

We surmise that if there exists a basic biological process common to prokaryotes and mammalian cells, it should be viewed as an SOS pathway: the lesions in mammalian cell DNA remaining after constitutive repair will induce a complex metabolic pathway that may lead to cell transformation, which is only one of many other phenomena produced. According to this hypothesis, lysogenic induction theoretically should provide a more reliable test for carcinogens than the mutagenicity test. In effect, mutagenesis may be produced by two different mechanisms, only one of which is going through the SOS pathway (Kondo 1974). In contrast, lysogenic induction is always a consequence of the induction of the SOS pathway.

To test this hypothesis, we have designed a new assay in which mutagenesis of prophage λ can be scored independently of lysogenic induction. Such a test can assess in the same treated cell whether a chemical induces either mutagenesis, or lysogenic induction, or both. We feel that a chemical that induces mutagenesis and lysogenic induction is a potential carcinogen.

MATERIALS AND METHODS

Most of the basic techniques used have been described in a previous paper (Moreau et al. 1976); the emphasis here is on some essential technical procedures.

Bacterial Strains

Strains used are described in the legends to the figures. A pedigree of the constructed strains will be sent upon request. The strain genetic markers relevant to this study are specified with a colon after the strain number. All strains were *mal*$^+$ *lam*^{s} (sensitive to phage λ) except GY5023: *mal lam*^{r}.

Lysogens have been constructed with λ wild-type phage, λ*cI857* and λ*13*. Phage λ*13* (L. Pereira da Silva and F. Jacob, pers. comm.) is a phage derived by recombination from λ*11 b2 v2 v3* (Jacob and Wollman 1954); it carries the *b2* region and is then able to lysogenize *E. coli* strains, although with a reduced efficiency.

Media and Cultures

LB consists of Difco yeast extract, 5 g; Difco tryptone, 10g; NaCl, 10 g; water, 1 liter. LBE consists of LB, supplemented with 0.2% glucose, and 20% medium E of Vogel and Bonner (1956). LA-Ca consists of LB, 1 liter supplemented with 0.2% glucose and 2.5 mM $CaCl_2$, and Biomar agar, 15 g. BT consists of peptone, 8 g; Difco tryptone, 5g; NaCl, 5 g; water, 1 liter. GT is BT with 12 g Biomar agar per liter. GT-amp is made of 500 ml of GT at 60°C mixed with 25 ml of BT medium to which 210 μl of a fresh solution of D-ampicillin in Me_2SO (25 mg/ml) has been added.

In all experiments, *envA* lysogens must be grown in LBE medium; as has been pointed out (Normark et al. 1969), mineral salts are required for the growth of *envA* bacteria. A fresh culture must be prepared as follows: an

overnight culture in LBE medium at 37°C is diluted 1:100 into fresh LBE medium and incubated with aeration at 37°C up to about 4×10^7 viable cells per ml ($OD_{650} = 0.3$). The exponential growth stage of a culture should not be prolonged by repeated dilutions into fresh LBE medium; this procedure can alter the *envA* cell viability.

Chemicals

Chemicals were dissolved and diluted when needed in dimethylsulfoxide (Me_2SO), spectrophotometric grade (Merck), and kept in the dark.

Aflatoxin B_1, aflatoxin G_1, and sterigmatocystin were from Makor; benzo(a)pyrene, benz(a)anthracene, and 7,12-dimethylbenz(a)anthracene from Fluka; 2-aminoanthracene, mitomycin C, and ethyl methanesulfonate from Sigma; 3-methylcholanthrene and sodium azide from Prolabo; methyl methanesulfonate from Eastman Kodak. Benzo(e)pyrene was kindly provided by P. Jacquignon, benzo(a)pyrene 4,5-oxide by D. M. Jerina.

NADP and glucose-6-phosphate were from Sigma, and D-ampicillin was donated by Bristol Laboratories.

Metabolization of Chemicals

Except when stated otherwise, the metabolizing mixture consisted of 1 volume of postmitochondrial fraction (Garner et al. 1972) and 6 volumes of NADPH-generating system: 150 mM phosphate buffer (pH 7.5), 3 mM NADP, 10 mM glucose-6-phosphate, and 12 mM $MgCl_2$. The latter was sterilized by filtration and stored at 4°C.

For brevity, the postmitochondrial fraction is called "microsomes" in this paper. Microsomes were from rats that had been submitted to one of the following treatments in the days prior to sacrifice: (1) three daily injections of 75 mg/kg of phenobarbital sodium (PB), (2) two daily injections of 20 and 10 mg/kg of 3-methylcholanthrene (MC), and (3) a single injection of Aroclor 1254 (Aro) (Ames et al. 1975). The type of microsomes used for each chemical tested is given in Table 1 (see below).

Measurement of Lysogenic Induction

A λ lysogen, when induced, gives rise to an infective center and, after a latent period of 45 minutes, bursts out and liberates free phage. Both free phage and infective centers are detected as plaques on a lawn of indicator bacteria.

In all the experiments described in this paper, lysogenic induction was measured by the number of induced infective centers. Treated λ lysogens in the latent period were plated onto GT-amp plates with 3 ml (or 2.5 ml in inductests I and II) of soft agar and with 0.2 ml of indicator bacteria GY4015 at about 2×10^9 cells per ml. Plates were incubated overnight at 37°C. Ampicillin was added in GT medium because this antibiotic prevents the noninduced bacteria from multiplying and therefore from releasing phage during the overnight incubation of plates; yet, ampicillin does not inhibit the induced lysogens from forming infective centers (Ogawa and Tomizawa 1967; Moreau et al. 1976).

The efficiency of lysogenic induction was calculated as the ratio of the number of induced infective centers to the number of original viable lysogens.

Inducing Activity of a Chemical

The induction curve as a function of the input concentration of an inducing chemical is usually composed of four parts (see Fig. 2b). The first flat line (part 1) corresponds to the background of spontaneously produced infective centers. At the minimal inducing concentration, the ascending part of the curve (part 2) begins, and with a two- or one-hit kinetics will finally reach its maximal value. This value plateaus for a wide range of concentrations (part 3). At toxic levels, the induction curve falls (part 4), the capacity of the cells to reproduce the induced prophage being affected. The minimal inducing concentration of a chemical is taken as an index of its inducing activity.

The Inductests

In an inductest the following are used: (a) the tester bacteria, such as *envA uvrB* (λ) lysogenic cells; (b) the λ indicator Amp^R bacteria to reveal the phage produced by induction; (c) the drug to be tested dissolved in Me_2SO; (d) if required, the metabolizing mixture that transforms the drug into active products; and (e) GT-amp plates and soft agar. Three variants of the test were designed which differ with respect to how and when items (a) through (d) are mixed.

INDUCTEST I: A QUALITATIVE SPOT TEST. The carcinogen is spotted directly onto plates or onto paper filters; the soft agar contains the λ lysogenic cells, the phage λ indicator bacteria, and the metabolizing mixture. Practically, 1 volume of tester lysogenic bacteria at about 8×10^3 cells per ml is mixed with 7 volumes of metabolizing mixture; 1-ml aliquots are poured onto GT-amp plates with 2.5 ml soft agar and with 0.2 ml GY4015 indicator bacteria at about 2×10^9 per ml. The tested chemical is spotted onto agar when it is hardened. As soon as metabolization of the drug occurs, metabolites will diffuse in the soft agar and will penetrate and induce the λ lysogens; plaques appear around the spots after overnight incubation at 37°C. The drug diffusing out of a spot forms concentric rings of decreasing concentrations.

INDUCTEST II: A QUANTITATIVE TEST ON PLATE. Five hundred to 1000 lysogenic cells are mixed with definite amounts of a chemical, the metabolizing mixture, and the indicator bacteria and are then poured onto GT-amp plates. In practice, 1 volume of lysogenic tester bacteria at about 4×10^3 cells per ml is mixed with 7 volumes of metabolizing mixture; 1-ml aliquots are distributed into test tubes containing 20 μl of Me_2SO with increasing amounts of the tested chemical. Preparations are then poured onto GT-amp plates with 2.5 ml soft agar and with 0.2 ml GY4015 indicator bacteria. Prophage λ induction is measured as infective centers formed after overnight incubation at 37°C.

INDUCTEST III: A QUANTITATIVE TEST IN LIQUID MEDIUM. A definite number of lysogenic cells (500–1000) and given amounts of a carcinogen and the metabolizing mixture are mixed and incubated at 37°C. Metabolization of the drug and cell penetration of the metabolites are stopped by dilution in soft agar and plating. Practically, a sample of lysogenic tester bacteria at about 2×10^4 cells per ml and the metabolizing mixture are mixed as de-

scribed above; 200-μl aliquots are distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of the tested chemical. Each preparation is gently agitated at 37°C in the dark for 15 or 25 minutes and then poured onto GT-amp plates with 3 ml soft agar and with 0.2 ml GY4015 indicator bacteria. Prophage λ induction is measured as infective centers formed after overnight incubation at 37°C.

RESULTS

Sensitive Tester Lysogens

It has been shown previously that the combination in lysogenic cells of *envA* and *uvrB* mutations increases the detection of aflatoxin B_1 metabolites more than 4000 times (Moreau et al. 1976). The *envA* mutation augments the sensitivity of the induction test 100-fold (Fig. 1). The fact that the *uvrB* mutation also increases cell sensitivity to aflatoxin B_1 (Fig. 2b) and benzo(a)pyrene metabolites (data not shown) indicates that these metabolites bind covalently to DNA and that the induced lesions are removed by the pyrimidine dimer excision repair pathway. In the case of benzo(a)pyrene, the absence of excision repair has two effects: prophage λ is induced at lower concentrations, and the efficiency of induction is increased from 10% to about 20%. The reported data were obtained with *mal envA* lysogenic cells.

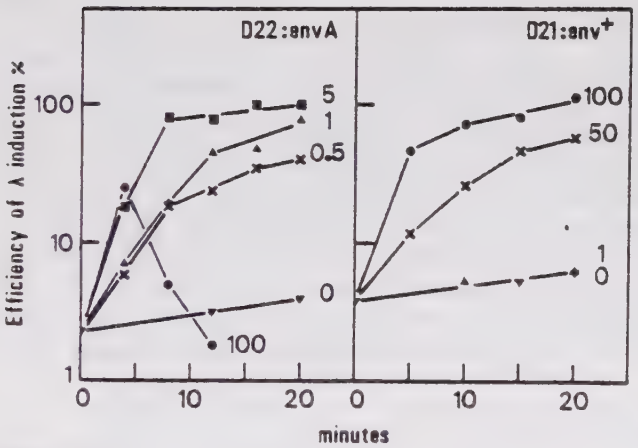


Figure 1

Kinetics of production of phage λ infective centers in *envA* bacteria as a function of the aflatoxin B_1 concentration. To the mixture of 600 μl of NADPH-generating system and 300 μl of microsomes (PB) were added 200 μl of bacteria D22:*envA* or D21:*env*$^+$ at about 4×10^7 cells per ml and then 25 μl of Me_2SO containing aflatoxin B_1. The final concentration of aflatoxin in μg/ml is indicated on each curve. Colony formers were assayed just before the addition of aflatoxin B_1. Each preparation was then incubated with aeration in the dark at 37°C. At the times indicated on the abscissa, diluted samples in 10 mM $MgSO_4$ were assayed for infective centers. Induction of prophage λ is expressed on the ordinate as the percentage of bacteria giving rise to infective centers.

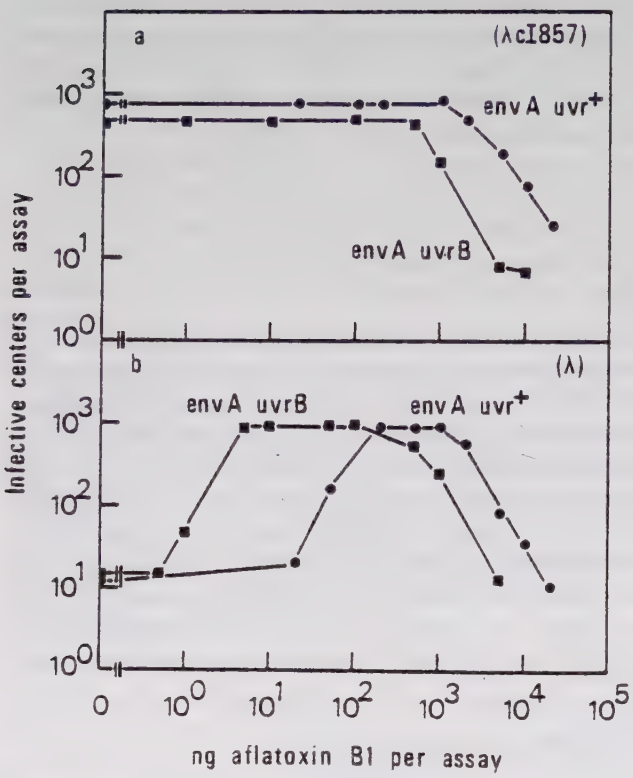


Figure 2

Capacity of the cells treated with aflatoxin B_1 to reproduce a thermally induced prophage. (*a*) One volume of bacteria GY5024:*envA uvr+* (λ*cI857*) or GY5029:*envA uvrB* (λ*cI857*) at about 2×10^4 cells per ml was mixed at 32°C with 7 volumes of metabolizing mixture; 200-μl aliquots were distributed into test tubes containing 5 μl of Me_2SO with increasing amounts of aflatoxin B_1 (indicated on the abscissa). Each preparation was gently agitated at 32°C in the dark for 25 min and then poured onto GT-amp plates with 3 ml soft agar and with 0.2 ml GY4015 indicator bacteria at 2×10^9 per ml. The development of the induced prophage was measured as infective centers formed after overnight incubation at 42°C. (*b*) Bacteria of strain GY5023:*envA uvr+* (λ) or GY5027:*envA uvrB* (λ) were treated as described above, except that incubation was for 15 min at 37°C (inductest III). Infective centers plotted on the ordinate were measured after overnight incubation at 37°C.

This relatively low efficiency of induction seems to be due to a superimposed alteration of the cell envelope of the tester lysogenic cells (see below).

Kinetics of Appearance and Disappearance of Active Metabolites

When lysogenic cells are mixed with the metabolizing mixture and a chemical at optimal inducing concentration, the rate of appearance of the induced infective centers as a function of time is the result of three processes. These

are (1) the activation and (2) the deactivation of the chemical (see next section) and (3) the penetration of the active metabolites into the lysogens. The third process is dependent upon the chemical structure of the metabolites and upon the bacterial cell envelope physiology.

Production and penetration of the active metabolites of aflatoxin B_1 can be very fast. As shown in Figure 1, in less than 10 minutes of incubation in liquid most of the treated lysogenic cells were induced. In contrast, when water-insoluble chemicals were tested, e.g., polycyclic hydrocarbons, infective centers seemed to be induced at a slow rate (data not shown); this is interpreted as essentially due to a slow rate of penetration of the metabolites into bacteria. This was much more pronounced when a *mal* mutation, which alters the bacterial cell envelope, was introduced into the lysogenic tester bacteria. With *mal envA uvrB*, the minimal inducing concentration of aflatoxin B_1 and of benzo(a)pyrene in inductest II was two times higher than with *mal*$^+$ *envA uvrB* tester bacteria. More significantly, the frequency of cells induced by benzo(a)pyrene was reduced from 100% to 20%; yet the *mal* mutation did not affect the efficiency of induction produced by aflatoxin B_1. The slow rate of penetration into cells of benzo(a)pyrene metabolites was even more visible in inductest III. After 15 minutes of incubation in liquid, only 3% of the treated GY5023:*mal envA* cells were induced by benzo(a)pyrene, whereas 100% were induced by aflatoxin B_1, aflatoxin G_1, or sterigmatocystin (data not shown).

When there is a slow cell penetration of the active metabolites, they have a chance to be deactivated before they can go through the cell's penetration barrier (see next section).

In the experiment reported in Figure 1, the efficiency of induction by aflatoxin B_1 metabolites reached a maximal value at between 10 to 15 minutes of incubation at 37°C. This means that aflatoxin B_1 active metabolites are short-lived. To obtain a more accurate estimate of the kinetics of disappearance of these metabolites, the metabolizing mixture and aflatoxin B_1 were first incubated at 37°C, then, at various times, *envA* lysogens were added and left in contact with the whole mixture for 15 minutes. The concentration of active metabolites varying as a function of time was determined by the efficiency of prophage λ induction. The concentration of active metabolites decreased almost exponentially. The half-life of active metabolites of aflatoxin B_1 was about 10 minutes under these conditions.

Metabolization of Carcinogens

Most foreign compounds that enter the body of a mammal are metabolized so as to be excreted rapidly (detoxification process) (for review, see Magee 1974). The enzyme systems involved in these reactions have been resolved (West and Lu 1972); their characteristic properties are that they (1) are found in high amounts in liver microsomes, (2) require NADPH and oxygen, and (3) are induced by various drugs.

In the detoxification pathway of certain chemical compounds, some metabolic intermediates are produced whose reactivity with cellular macromolecules, unfortunately, is increased rather than decreased. This is the case for

many chemicals whose original molecules are not active per se but become carcinogenic after metabolic activation in vivo (Miller and Miller 1971 and this volume).

Bacteria do not possess such detoxification enzymes but become sensitive to chemical carcinogens that have been activated by mammalian liver homogenates (microsomes). The active metabolites formed in vitro are revealed by their toxic (Garner et al. 1972) and mutagenic properties (Garner and Wright 1973; Ames et al. 1973), as well as by their lysogenic inducing activity (Goze et al. 1975; Moreau et al. 1976).

When a chemical is activated in vitro, three parameters are crucial to obtaining optimal results: (1) the origin of the microsomes, e.g., the animal species, the tissue employed to make homogenates, the physiological state of the animals (pretreatment by drugs, diet); (2) the concentration of microsomes used in the assay; and (3) the type of incubation, i.e., in liquid or in soft agar (Saito et al. 1975; Ames et al. 1975; Montesano and Bartsch 1976; Bartsch 1976; Sugimura et al. 1976).

We have routinely used rat liver microsomes to activate chemicals. We are aware of the fact that homogenates from other tissues or tissues from other animals might be more efficient. The liver is only one of the target organs to be considered and animals other than the rat may be more sensitive to a given chemical (Montesano and Bartsch 1976; Bakshi et al., this volume).

To stimulate the activation of the chemicals assayed in the inductests, we have used microsomes from rats treated with one of the following drugs known to have different specificities as enzyme inducers (Conney 1967; Ames et al. 1975): 3-methylcholanthrene (MC), which is essentially an inducer of benzpyrene metabolism; phenobarbital (PB), which stimulates the metabolism of other classes of substrates; and Aroclor 1254 (Aro), which seems to stimulate the metabolism of the two classes of compounds.

As expected, in the case of benzo(a)pyrene, similar results were obtained with MC or Aro microsomes (see Fig. 4 and Table 1 below), whereas PB microsomes were inactive. For aflatoxin B_1, microsomes were equally efficient whether or not they had been induced by PB or MC (data not shown). However, at high concentrations of aflatoxin B_1, the capacity to reproduce the phage was much more affected when PB microsomes were used. This is in agreement with previous observations that highly toxic metabolites of aflatoxin B_1 are produced with this type of microsomes (Garner et al. 1972).

Another important point to consider in order to obtain optimal results is the concentration of the microsomes used in an assay. Figure 3 shows that, in the case of lysogens treated with aflatoxin B_1, a nearly optimal rate of metabolization was obtained when the metabolizing mixture used consisted of 3 volumes of PB microsomes to 6 volumes of NADPH-generating system (300 μl/600 μl). However, a similar efficiency of induction was obtained when lower volumes of microsomes in the range of 0.5 to 3 volumes (50–300 μl) were used. The same results were obtained in inductest II. In contrast, for benzo(a)pyrene, the concentration of microsomes was crucial: the optimal mixture consisted of 0.5 to 1 volume of Aro microsomes to 6 volumes of NADPH-generating system. Higher amounts decreased the sensitivity of the test (Fig. 4). This result suggests that, at high concentrations of microsomes, the reactions of metabolization are very rapid (see

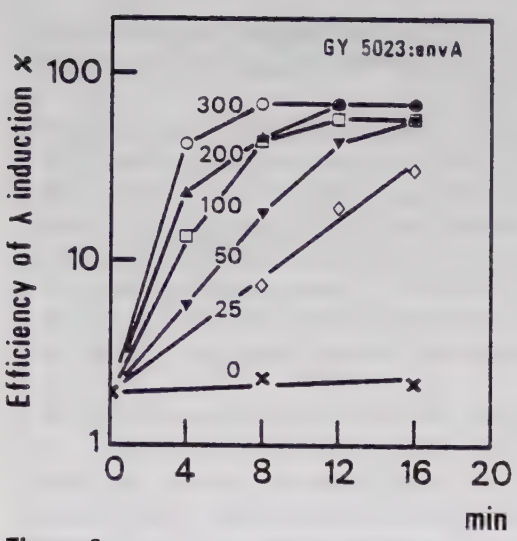


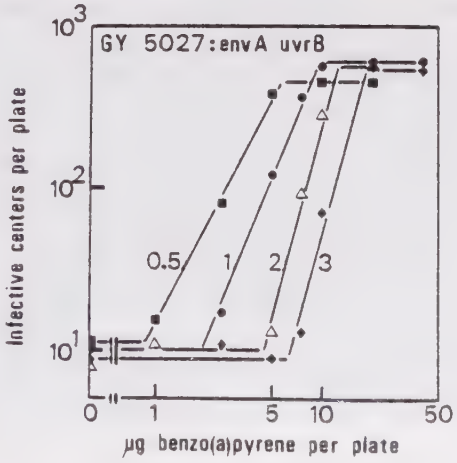
Figure 3
Kinetics of prophage λ induction by aflatoxin B_1 as a function of the concentration of microsomes. GY5023:*envA* (λ) bacteria were treated with aflatoxin B_1 as described in the legend to Fig. 1, except that the variable volumes of PB microsomes were all adjusted to a 300-μl final volume with LBE. The concentration of aflatoxin B_1 in each preparation was 5 μg/ml.

Fig. 1): the active metabolic intermediates are further metabolized and then deactivated before they can penetrate the lysogens.

Toxicity Test for Chemical Carcinogens

All the known inducers of prophage λ have two antagonistic effects: (1) they trigger the multiplication of the prophage, and (2) they have a toxic effect so

Figure 4
Prophage λ induction by benzo(a)pyrene as a function of the concentration of microsomes. GY5027:*envA uvrB* (λ) bacteria were treated with benzo(a)pyrene as described in Materials and Methods, Inductest II, except that increasing concentrations of Aro microsomes were used. The metabolizing mixture used was composed of 6 volumes of NADPH-generating system mixed with 0.5 to 3 volumes (indicated on the graph) of microsomes.



that the capacity of the treated cells to reproduce the induced phage decreases. This happens to be the case at concentrations over 100 μg/ml in the experiments described in Figure 1.

The range of toxicity of a chemical can be determined by using lysogenic cells carrying the thermally inducible λ*cI857* prophage. In effect, when a culture of lysogens carrying this mutant prophage is submitted to a chemical at 32°C (at which temperature the mutant prophage is not inducible) and then shifted to 42°C, every lysogenic cell is induced. Therefore when one observes a reduction in the number of λ*cI857* infective centers formed, it means that the tested chemical produces a deleterious effect on the cells, which are no longer able to reproduce the induced phage or to form an infective center.

In Figure 2a, we see that, as expected, at low concentrations of aflatoxin B_1, 100% of the λ*cI857*-treated lysogens produced infective centers. At higher concentrations, the number of λ*cI857* infective centers declined sharply. This shows the toxic effect of the aflatoxin metabolites. The descending portions of the curves of the thermally induced prophage (Fig. 2a) are parallel to those of the corresponding λ wild-type lysogens (Fig. 2b). Note that the minimal inducing concentration of aflatoxin B_1 was sharply lowered in *uvrB* lysogens. This shows that the target for lysogenic induction is DNA on which the *uvrB* product operates. In contrast, the cell's capacity to reproduce phage λ, which depends on the integrity of the RNAs and enzymes, was not much more affected in *uvrB* than it was in *uvr*$^+$ lysogens.

Inducing Activity of a Chemical vs. Its Carcinogenic Activity

For many chemicals, there is general agreement about their carcinogenic potency. For some others, unfortunately, there are some conflicting opinions as to whether or not they are carcinogenic. Many authors differ in evaluating the carcinogenic potency of, say, benz(a)anthracene as compared to 7,12-dimethylbenz(a)anthracene. One can deplore that a carcinogenic potency index has not yet been defined which takes into account such parameters as (1) the dose of the chemical used, (2) its mode of administration, (3) the animal species treated, (4) the frequency of tumors produced, etc.

We have found for three series of chemicals a good correlation between their carcinogenic potency in rodents and their prophage-inducing activity in bacteria (Fig. 5; Table 1). Aflatoxin B_1 is a potent carcinogen as well as a potent λ inducer, whereas aflatoxin G_1 is weak in both cases. The same holds true for benzo(a)pyrene and benzo(e)pyrene. Benz(a)anthracene, a questionable carcinogen, does not induce prophage λ even though it is a potent mutagen (McCann et al. 1975); its carcinogenic derivative, 7,12-dimethylbenz(a)anthracene is a λ inducer as well as a mutagen.

There is also an excellent correlation between the carcinogenic potency of nitroquinoline-oxide derivatives and their inducing activity (Epstein and Saporoschetz 1968). Such a correlation extends to many other chemicals (for review, see Heinemann 1971; Yamamoto 1974).

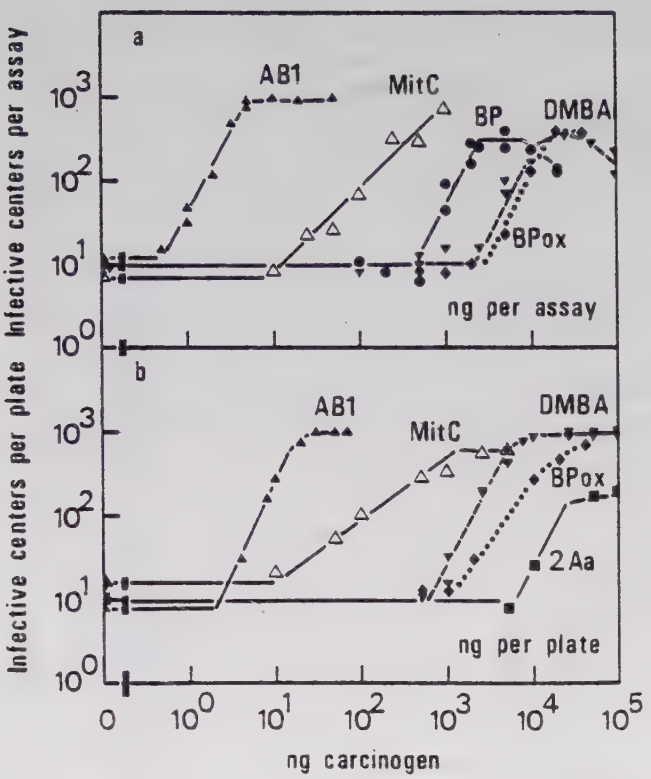


Figure 5

Prophage λ induction by various chemical compounds. (*a*) GY5027:*envA uvrB* (λ) bacteria were treated as described in Materials and Methods, Inductest III, with increasing amounts (indicated on the abscissa) of aflatoxin B_1 (AB_1) (▲); mitomycin C (Mit C) (△); benzo(a)pyrene (BP) (•); 7,12-dimethylbenz(a)anthracene (DMBA) (▼); or benzo(a)pyrene 4,5-oxide (BPox) (◆). (*b*) The same lysogenic cells (GY5027) were treated as described in Materials and Methods, Inductest II, with increasing amounts (indicated on the abscissa) of AB_1, Mit C, DMBA, BPox, or 2-aminoanthracene (2Aa) (▪).

Scoring Mutagenesis of Prophage λ Independently of Its Induction

We have developed a new assay in which we can assess whether, in the very same treated cell, a given chemical induces mutagenesis, or lysogenic induction, or both phenomena. The type of mutation scored renders prophage λ insensitive to its repressor; this is a forward mutation in the right operator of the immunity region of the prophage. As soon as the mutation arises, the prophage starts developing even in the presence of the λ repressor: there is no selection against such a mutational event. This type of prophage mutation can therefore be scored independently of lysogenic induction, i.e., independently of λ repressor inactivation.

We have chosen to assay prophage mutagenesis and not bacterial mutagenesis because, in the occurrence of lysogenic induction accompanying

Table 1
Inducing Activity vs. Carcinogenic Activity

Chemical compound	*Microsomes*	*Inducing activity*	*Minimal inducing concentration in μg*[a]		*Carcinogenic activity*
			inductest II	*inductest III*	
Aflatoxin B_1	PB	+	0.002	0.0005	+
Aflatoxin G_1	PB	+		0.01	+
Benzo(a)pyrene	MC	+	1	0.4	+
Benzo(e)pyrene	MC	−	(1–100)	(1–100)	−
Benzo(a)pyrene 4,5-oxide	—	+	1	3	±
Benz(a)anthracene	PB	−	(1–200)	(1–100)	±
7,12-dimethylbenz(a)anthracene	PB	+	0.5	2	+
2-Aminoanthracene	Aro	+	5		+
3-Methylcholanthrene	PB	+	8		+
Mitomycin C	—	+	0.01	0.01	+
Sodium azide	—	−	(0.1–100)		−

[a] Data are taken from Fig. 6 and from a previous publication (Moreau et al. 1976). Tester lysogenic cells used were GY5027:*envA uvrB* (λ). For chemicals that did not induce, the range of concentrations tested is given in parentheses.

mutagenesis, the mutated bacteria would be killed by the developing prophage.

An *envA uvrB* lysogen carrying prophage λ*13* has been constructed. Phage λ*13* is a *b2*$^+$ derivative of λ*11 b2 v2 v3* and can lysogenize a sensitive bacterium. The operator constitutive mutations *v2* and *v3* are in the left and right operators, respectively, of the immunity region. In spite of these mutations, λ*13* is still sensitive to the repressor action, it forms small turbid plaques on sensitive indicator bacteria, and it does not grow on λ lysogenic indicator bacteria. The appearance of a third mutation *v1*, also in the right operator, renders the phage insensitive to the repressor; the phage becomes virulent, i.e., it overcomes the immunity of a λ lysogen. The virulent mutants form small clear plaques on λ lysogenic, as well as on nonlysogenic, bacteria. This property provides an easy means of differentiating the mutant from the parental phage.

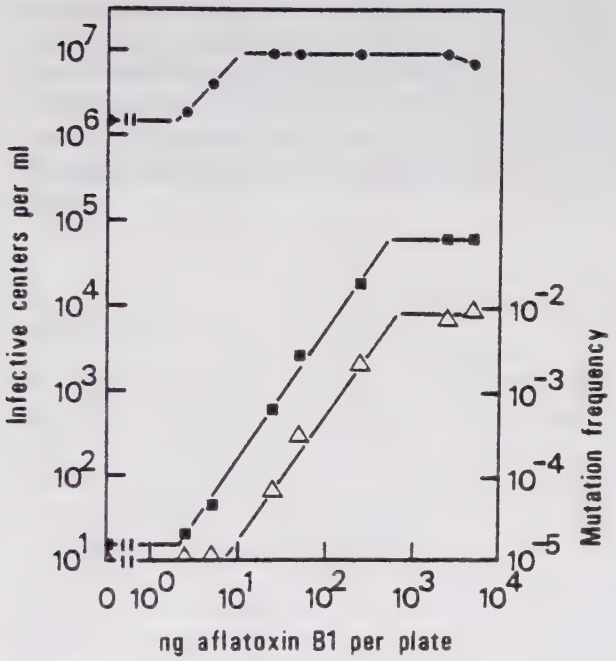


Figure 6

Mutagenic and inducing activities on prophage λ of aflatoxin B_1. (*a*) *Assay for induction.* Bacteria of strains GY5031:*envA uvrB* (λ*13*) were treated as in inductest II, with increasing concentrations of aflatoxin B_1 as indicated on the abscissa. (*b*) *Assay for mutagenesis.* One volume of bacteria of the same strain GY5031 at about 4×10^7, 4×10^6, or 4×10^5 cells per ml was mixed with 7 volumes of metabolizing mixture: 1-ml aliquots of each mixture were distributed into test tubes containing 25 μl of Me_2SO with increasing concentrations of aflatoxin B_1. Preparations were then poured onto GT-amp plates with 3 ml soft agar and with 0.15 ml GY3646 lysogenic indicator bacteria at 2×10^9 per ml. Mutagenesis (△) is expressed on the ordinate as the ratio of the number per ml of infective centers formed on GY3646 lysogenic indicator bacteria (▪) to the number of infective centers produced on GY4015 nonlysogenic indicator bacteria (•).

Lysogenic induction is always a consequence of the SOS pathway, but mutagenesis may not be. Mutations may arise by at least two different mechanisms (see Discussion). Therefore, one expects to find two broad categories of mutagens: mutagens that are lysogenic inducers and mutagens that are not. A representative of the first class is aflatoxin B_1; ethyl methanesulfonate (EMS) is a representative of the second class. This can be seen in Figures 6 and 7a. Aflatoxin B_1 metabolites increased phage mutagenesis 1000-fold over the background within the same range of concentrations which induce prophage λ. In contrast, EMS induced phage virulent mutations but not lysogenic induction. Methyl methanesulfonate (MMS) has a mutagenic action with a very slight inducing effect (Fig. 7b).

These results support the idea that potent carcinogens will all be found to be lysogenic inducers as well as mutagens, whereas mutagens that are not lysogenic inducers will be either noncarcinogenic, for example, 2-aminopurine and sodium azide, or feebly carcinogenic, for example, EMS and MMS.

Sodium azide, which is a mutagen as efficient as EMS in barley and induces base-substitution mutations in *S. typhimurium* (Nilan et al. 1973; McCann et al. 1975), has neither an inducing activity nor a mutagenic activity in our tests. Sodium azide seems to have an indirect action on DNA (Kleinhofs et al. 1975). The DNA of the λ operators are covered by the repressor molecules, which may protect these operators from being mutated by

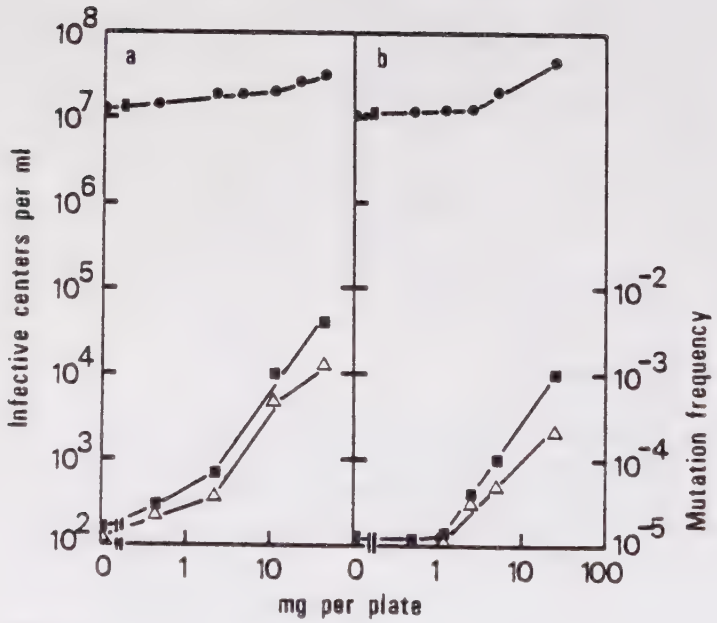


Figure 7

Mutagenic and inducing activities on prophage λ of EMS and MMS. Bacteria of strain GY5031 were treated as described in the legend to Fig. 6 with increasing concentrations of (*a*) ethyl methanesulfonate (EMS) or (*b*) methyl methanesulfonate (MMS).

sodium azide. It has been shown in vitro that methylating agents can methylate DNA covered by the repressor (M. Ptashne, pers. comm.). We find mutagenesis in vivo with EMS and MMS.

Toward a Biochemical Assay for Carcinogens

One of the virtues of a test for potential carcinogens that makes use of lysogenic induction is that most of the cells used in the assay undergo a dramatic change. Such a mass effect can therefore be measured at the biochemical level. In contrast, mutagenesis, which occurs in a small fraction of the cell population, is detected only by sensitive genetic techniques.

We have recently used (A. Levine, P. Moreau, R. Devoret, S. Adhya, and M. Gottesman, unpubl.) tester bacteria in which the galactose operon is under the control of the λ repressor. In these bacteria, lysogenic induction can be measured by the production of galactokinase. When such bacteria were treated with aflatoxin B_1 metabolites, galactokinase activity was turned on within an hour after the treatment. Such an assay has practical as well as theoretical implications. It takes only half a day to test potential carcinogens as lysogenic inducers. It also points to the fact that in a situation in which a viral repressor gene controls a chromosomal gene, a carcinogen, by producing the inactivation of a viral repressor, turns on the expression of a chromosomal gene.

DISCUSSION

Is it worthwhile to try to develop a lysogenic induction test for potential carcinogens? Since such a test involves a prokaryotic organism, one might fear that it would simply duplicate the *Salmonella* mutagenicity test (mutatest) which has already been used to test a great many potential carcinogens (McCann et al. 1975). We believe that a lysogenic induction test (inductest) for potential carcinogens is useful for practical as well as theoretical reasons.

From a practical viewpoint, the inductest has many qualities: (1) It is as sensitive as the mutatest. (2) There is a good correlation between the carcinogenic activity of chemicals and their inducing activity. (3) In the inductest, one can detect mutagenesis of the prophage as well as its induction in the very same treated lysogenic cell: two biological manifestations of the action of potential carcinogens can be scored in one assay. (4) The use of a thermoinducible prophage provides a toxicity test. (5) The fact that lysogenic induction occurs in most of the treated cells has led to the design of a biochemical assay that is rapid and easy. (6) The presence of potential carcinogens in body fluids can be assessed with the inductest more easily than with the mutatest. In effect, the presence of histidine in the body fluids does not hamper the inductest, whereas it may be a cause of technical difficulties in the mutatest. Detection of induction can be carried out in rich media containing much histidine.

Important theoretical questions are raised by the fact that carcinogens in-

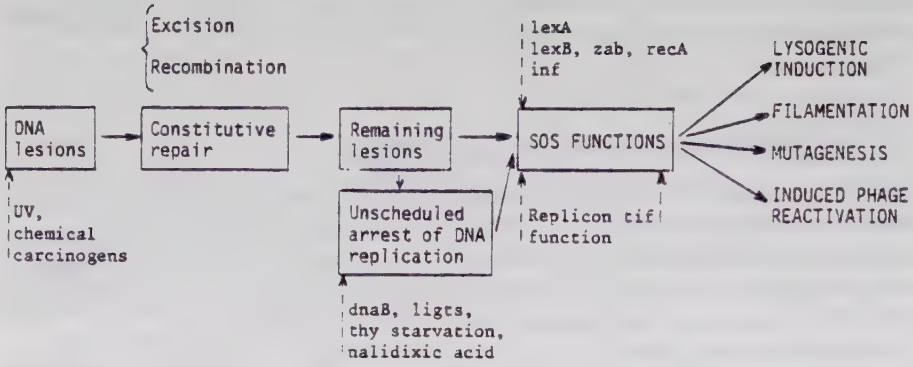


Figure 8
Causes and consequences of SOS functions in *E. coli*. Upward arrows indicate actions that increase, produce, or enhance; downward arrows are used for actions that decrease, suppress, or inhibit.

duce lysogenic bacteria. Is there a biological mechanism common to lysogenic induction and mammalian cell transformation? Before considering this question, we must first ask ourselves: What do we know about the course of events which take place in a prokaryote after its exposure to a carcinogen? Recent work in our laboratory (Goze et al. 1975; Sarasin et al. 1976; Devoret et al. 1976) and other laboratories (Witkin 1976; Radman et al., this volume) points to the following scenario (Fig. 8).

Carcinogens such as UV light and aflatoxin B_1 produce DNA lesions, which are subject to at least two constitutive repair mechanisms (Devoret et al. 1975). Since the efficiency of repair is never absolute, some residual lesions remain in the DNA. The cellular DNA replicates cyclically so that residual DNA lesions have to go through the DNA replicating machinery. DNA replication is impaired at the site of the lesion: there are gaps formed on the newly replicating strand opposite the lesions (Rupp and Howard-Flanders 1968). A cluster of DNA lesions has every chance to produce a profoundly altered DNA structure that causes an "unscheduled inhibition of DNA replication." This situation calls for cellular emergency processes since cell survival is at stake.

It appears that evolution has invented a complex cellular pathway to cope with this emergency situation. To ensure cell survival, some functions, which we call "SOS functions," are induced by the very same trouble at the replication fork that is likely to cause cell death. The SOS functions (see Fig. 8) are expressed by a set of genes or loci, which in *E. coli* are designated *recA*, *lexA*, *lexB*, *zab*, *tif*, etc. Although the products of these loci have not yet been characterized, their coordinate action in a biochemical pathway (Witkin 1974), which we call "SOS pathway," leads to such phenomena as:

1. *Error-prone repair*—as exemplified by reactivation and mutagenesis of UV-damaged phage (induced phage reactivation) or repair and mutagenesis of the damaged cell itself. Error-prone repair has been designated by Radman (1975) as SOS repair; note that this process is only one of

the manifestations of a more general cellular process, the SOS pathway.

2. *Cell filamentation*—the result of absence of septum formation which might allow recombination between two damaged nuclei and therefore might lead to the restoration of a functional chromosome.
3. *Lysogenic induction*—a consequence of the fact that the repressor coded by the prophage is irreversibly inactivated: the prophage develops into a vegetative phage, thus escaping from a cell almost doomed to die. In this case, it is not the survival of the bacterial cell, but the survival of the phage, which is ensured by the SOS pathway. Temperate phages have evolved to recognize when SOS functions are induced: their repressors are sensitive to a cellular inducer, which results from the expression of the SOS functions. Many other biological phenomena that are dependent on the expression of the SOS functions have been reviewed recently by Swenson (1976) and Witkin (1976).

Witkin (1976) has proposed that error-prone repair and filament formation are normally prevented from occurring by specific repressors analogous to the λ repressor. She has postulated that the molecular mechanism of the SOS functions is the coordinated inactivation of such repressors. The current idea is that inactivation of repressors might result from protease action. Until now, this has only been established for the λ phage repressor (Roberts and Roberts 1975).

The factual correlation between carcinogenesis in mammals and mutagenesis and lysogenic induction in prokaryotes implies that there must exist a basic biological process common to prokaryotes and mammalian cells. This has led us, in collaboration with others (Sarasin et al. 1976; Moreau et al. 1976; Devoret et al. 1976), to hypothesize that, in mammalian cells, the DNA lesions remaining after constitutive repair induce an SOS pathway analogous to that occurring in prokaryotes; one of the many long-term biological consequences of the induction of the mammalian cell SOS pathway would be cell transformation.

A phenomenon similar to lysogenic induction occurs in mammalian cells. Two types of transformed cell lines have been described which appear to behave as if they were inducible lysogens. These are a polyoma-virus-transformed cell line and a simian-virus-40-transformed cell line. A high titer of polyoma virus is induced by treatment of the transformed cell line with mitomycin C, polycyclic hydrocarbons, and radiations (Fogel and Sachs 1970; Huberman and Fogel 1975; Huberman, this volume). Simian virus 40 is similarly induced by radiations and chemical treatment of the transformed cell line (Kaplan et al. 1975). Moreover, murine leukemia viruses have been induced in AKR and BALB/3T3 cells by 5-IdU or 5-BrdU (Lowy et al. 1971; Aaronson et al. 1971). These experiments provide support for the theory that there may be an SOS pathway in mammalian cells leading to derepression of some as yet unidentified functions. An additional argument in favor of our scheme is provided by the fact that protease inhibitors have been shown to prevent tumor formation (Troll et al. 1975), namely, in the system of C3H/10T1/2 mouse cells (T. Kuroki, pers. comm.).

The idea of the existence of mammalian SOS functions raises another

question: Which steps of the two SOS pathways are similarly affected in *E. coli* and in mammalian cells? Are the DNA lesions produced by the common factor or are there further homologous steps?

Most chemical carcinogens produce DNA lesions. It is very likely that the in vivo active forms of a carcinogen are identical to those generated in vitro by mammalian enzymes. If this is so, DNA lesions induced in mammalian cells by carcinogens should be similar to those induced in prokaryotes. Does any assay for potential carcinogens using prokaryotes serve only as a probe for DNA lesions? This does not seem to be strictly the case.

Some chemicals, such as 5-fluorouracil (Marcovich and Kaplan 1963), trimethoprim (Hitchings 1973), and nalidixic acid (Simon et al. 1974), cause an unscheduled inhibition of DNA replication; they induce SOS functions in bacteria, although they do not produce any primary DNA lesions (see below). Moreover, one can imagine that other chemicals might act further along the SOS pathway either as an activator of a protease or directly by binding to some cellular repressors.

With all the above-mentioned considerations in mind, one can deduce that lysogenic induction should theoretically provide a more faithful test for carcinogens than the mutagenicity test. In effect, lysogenic induction is one of the expressions of the SOS pathway in prokaryotes, whereas mutagenesis may not always be. Mutagenesis can be produced by at least two different mechanisms (Kondo 1974), one of which does not require the expression of the SOS pathway. One can cite as an example the case of 2-aminopurine, a base analog, which is mutagenic in bacteria by inducing mispairing (Vigier and Marcovich 1973). This compound does not induce prophage λ (Devoret and Blanco 1970) and is not known to be carcinogenic. The same can be said about many other chemicals such as sodium azide and ethyl methanesulfonate, which are potent mutagens in the *Salmonella* test system, do not induce prophage λ, and are not carcinogenic or are very feebly so. An explanation of these facts can be given by assuming that the chemicals cited above produce small, discrete DNA alterations. These alterations may not be "felt" as DNA lesions (case of 2-aminopurine) and therefore do not trigger the SOS functions. If the alterations produced are repaired (case of EMS), repair is of the short-patch type (Cooper and Hanawalt 1972) which does not involve the *recA* and *lexA* functions. When the long-patch repair type, which requires the SOS functions, operates, there is a correlation between the mutagenic, inducing, and carcinogenic properties of the chemical tested.

Like the mutatest, the inductest is not exempt from giving positive results for some chemicals that do not appear to be carcinogenic. Chemicals that produce in *E. coli* unscheduled inhibition of DNA replication, such as nalidixic acid (Cowlishaw and Ginoza 1970), trimethoprim (A. Goze, pers. comm.), or 5-fluorouracil (Marcovich and Kaplan 1963), induce prophage λ; these chemicals mimic the effect of thymine deprivation (Sicard and Devoret 1962). The reason these chemicals, used extensively as therapeutic agents, may not be carcinogenic can be clearly seen in the case of trimethoprim, whose target enzyme is dihydrofolate reductase. Binding of trimethoprim to dihydrofolate reductase is species-dependent. The affinity of trimethoprim for this enzyme decreases as a function of the evolutionary level of the organism studied. In *E. coli*, 0.5×10^{-8} M of trimethoprim inhibits 50% dihydrofolate reductase,

whereas, in human liver cells, the same inhibition is obtained with a 60,000-fold higher concentration of trimethoprim (Burchall 1973). In short, in mammalian cells, trimethoprim does not affect DNA synthesis much. An analogous situation is found in the case of nalidixic acid. Experiments performed to see whether this compound would block cell division of T lymphocytes induced by phytohemagglutinin were negative, whereas methotrexate used as a control did block cell division (M. Croze, pers. comm.). Noncarcinogenic chemicals that are positive in the inductest raise an interesting problem of comparative cell physiology.

If one excludes the case of chemicals whose cellular action decreases from prokaryotes to higher eukaryotes as a function of the evolutionary level of the cell considered, one is struck by the close correlation between the carcinogenic activity of chemicals and their prophage-inducing activity. This must reflect some basic cellular mechanisms which are present in cells all along the evolutionary scale. Our explanation is that cell transformation of mammalian cells results from a two-stage process: (1) cellular SOS functions are induced through some specific signal provided by an altered replication of the chromosome, and (2) the SOS functions cause the irreversible depression of some proviral genes, perhaps through the irreversible inactivation of some regulatory proteins by a protease action.

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EXPRESSION OF A BACTERIAL GENE TURNED ON BY A POTENT CARCINOGEN

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Summary

Contrary to mutagenesis, lysogenic induction produced by chemical carcinogens occurs in the majority of a population of lysogenic cells. Such a mass effect can therefore be measured at the biochemical level using an *E. coli* tester strain in which the galactose operon has been put under the negative control of the λ repressor. In this publication we show that galactokinase synthesis is turned on by aflatoxin B1 metabolites within an hour after treatment of the tester bacteria. Such a biochemical assay provides a useful means for identifying potential chemical carcinogens.

Introduction

In a recent publication, Moreau et al. [19] described a test for potential carcinogens called the "Inductest" since it is based upon the induction of prophage λ in *Escherichia coli* K12. In the classical techniques of mutation testing [4] mutagenesis is measured in at most 10^{-3} cells in a population whereas in the inductest most of the treated lysogenic cells undergo a dramatic change: they lyse and release free phage. Such a mass effect should be amenable to detection in a biochemical assay. We describe here a method whereby potential carcinogens can be detected within 2 h by measuring the induction of the synthesis of galactokinase. We have employed a strain of *E. coli* K12 in which the galactose operon is integrated into an early transcribed region of prophage λ and is thus directly *under the negative control of the λ repressor*. Induction of prophage λ leads therefore to the synthesis of galactokinase as demonstrated with the use of aflatoxin B1, a potent hepatocarcinogen for several species probably including man [28].

Materials and methods

Bacterial strains

Strain SA 1511 was derived from *E. coli* strain M72: F^-, sup^0, *strA* and lysogenic for λ*cI857* [1]. First, an inactivating mutation (*P10*) was introduced into the promoter of the *gal* operon. Second, the *gal* operon was brought closer to the prophage P_L^+ promoter by introduction of the deletion Δ312; such a deletion, covering the region from the bacterial gene *chlD* to the phage gene *cIII*, increases the level of read-through *gal* transcription from the P_L^+ promoter [2]. In the final step, a second deletion ΔH1 [6], removing the prophage gene *tof* and the bacterial gene *uvrB* making the cell sensitive to UV-light, was introduced into the strain. Deletion of the phage *kil* gene [12] by Δ312, and deletion of the phage replication genes *O* and *P* by ΔH1, render the cells able to survive lysogenic induction. Strain SA 1511 carries an intact N^+ gene, which prevents termination of the transcription initiated at the prophage P_L^+ promoter site [2,8,9].

Strain SA 1512 has the same genetic features as SA 1511 except that it carries a wild-type phage repressor gene introduced by recombination with λcI^+.

Fig. 1. An abbreviated map of prophage λ (from the left *att* to the right *att*) and of the neighboring bacterial genes in strain SA 1512. The *gal* operon (left end of the map) consists of three structural genes, *K*, *T* and *E*, with the promoter *P* located at the *E* end. The promoter carries the *P10* mutation. The solid bars signify the extents of the two deletions Δ312 and ΔH1. The wavy line with an arrow-head indicates the *gal* transcription after lysogenic induction.

Chemicals

NADP, glucose-6-phosphate, and ATP were from Sigma; [^{14}C]galactose from C.E.A.; aflatoxin B1 (AB1) from Makor. DEAE (DE 81) and glass-fiber paper discs (GF/C) were from Whatman.

Aflatoxin B1 was dissolved in dimethylsulfoxide, spectrophotometric grade (Merck), and stored at −20°C.

Media

LB consists of Difco yeast extract, 5 g; Difco tryptone, 10 g; NaCl, 10 g; water, 1 l.

Treatment of tester bacteria with activated aflatoxin B1

As with most chemical carcinogens, aflatoxin B1 is not active per se, but becomes carcinogenic after metabolic activation in vivo [17,18]. Activation of aflatoxin B1 in vitro and cell treatment were performed essentially as previously described [19,20]. Bacteria were grown in LB medium at 37° (or at 32° in the case of λ*cI857* lysogens) to 2×10^8 cells per ml, then centrifuged and concentrated 10-fold in the same medium; 0.1-ml aliquots were then mixed with 0.1 ml of rat-liver homogenate (9000 × *g* supernatant) [10], 0.6 ml of NADPH-generating system (150 mM phosphate buffer, pH 7.5; 3 mM NADP; 10 mM glucose-6-phosphate; 12 mM $MgCl_2$), and 25 μl of dimethylsulfoxide

containing increasing amounts of aflatoxin B1. Reaction mixtures were incubated at 32° in the dark for 30 min and then centrifuged at 4° for 10 min at 4000 × *g* to eliminate the rat-liver homogenate. Cells were washed with cold LB medium, resuspended in 0.6 ml of warm LB medium and incubated further at 37° or first at 42° for 20 min and then at 37° in the case of λ*cI857* lysogens. At various times, aliquots were taken and assayed for galactokinase activity.

Galactokinase assay

The assay was essentially the same as that described by Sherman [25]; modifications were from O. Reyes (personal communication).

The principle of the test is to measure the activity of galactokinase which converts [^{14}C]galactose into [^{14}C]galactose-1-phosphate in the presence of ATP. The phosphorylated product was specifically retained on an anion-exchange filter and measured by liquid-scintillation counting.

A 100-μl suspension of AB1-treated bacteria were lysed by the addition of 10 μl toluene and 20 μl of 7 mM EDTA, 330 mM β-mercaptoethanol, 0.6 mg/ml chloramphenicol and 0.25 mg/ml rifampicin. After incubation at 32° for 10 min, 50 μl of each lysate were mixed with 20 μl ATP (1.6 mM), 20 μl NaF (3.2 mM) to inhibit bacterial phosphatases, and 10 μl [^{14}C]galactose (0.2 μCi). Mixtures were incubated at 32° for 10 min and chilled. Conversion of galactose to galactose-1-phosphate occurred with linear kinetics during this period. Two 25-μl samples were spotted onto Whatman DE 81 discs. Before the spots had dried the discs were washed three times in distilled water, dried, and counted in a scintillation fluid composed of 4 g Omnifluor (NEN Chem. GmbH) per liter of toluene. A control without extract was done under the same conditions; its value was subtracted from the experimental results. Total radioactivity was measured by spotting 25 μl of each reaction mixture onto glass-fiber paper discs. One unit of galactokinase converts 1 μmole galactose into galactose-1-phosphate per hour.

Results

Rationale of the test

In order to measure prophage λ induction rapidly, we took advantage of the fact that transcription initiated from the P_L^+ promoter of λ (see Fig. 1) is resistant to termination and under appropriate conditions extends into genes to the left of the prophage such as the *gal* operon [1]. Thus, inactivation of the λ repressor by exposure to inducing treatments can be followed by monitoring the "escape synthesis" of one of the *gal* enzymes, namely galactokinase, the product of the *galK* cistron. Potential carcinogens that induce prophage λ [19] might therefore be tested by this biochemical assay.

Activated aflatoxin B1 induces the synthesis of galactokinase

Using prokaryotes [10] or cultured human cells [24], it has been demonstrated that pure aflatoxin B1 is inactive, whereas aflatoxin B1 incubated with a metabolizing mixture containing rat-liver microsomal enzymes affects cellular metabolism. Fig. 2 shows that only activated aflatoxin B1 induced galactokinase synthesis in the tester strain SA 1512, whereas aflatoxin B1 alone did not. At

32

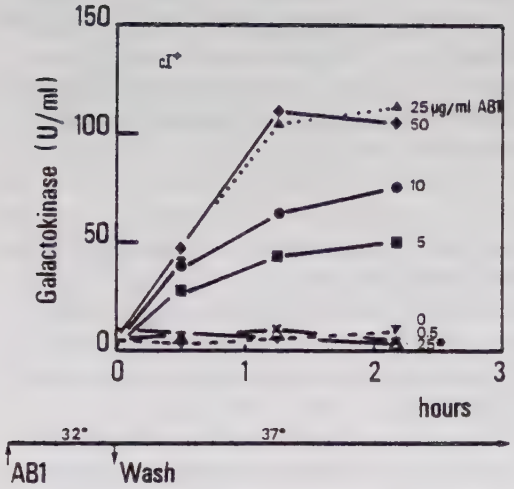

Fig. 2. Induction of galactokinase by activated aflatoxin B1. Bacteria SA 1512 were treated with increasing concentrations of aflatoxin B1 (indicated on the figure) as described in Materials and Methods except in one case (25 µg/ml *) (△) in which the activation mixture was omitted. Bacteria were then incubated at 37° and at times indicated on the abscissa, samples were assayed for galactokinase activity.

optimal inducing concentration (25 µg/ml) the production of the enzyme was enhanced about 20-fold over the background. Moreover, a linear dose—response curve was obtained between 0.5 to 25 µg/ml of aflatoxin B1.

Synthesis of galactokinase was induced within the expected range of concentrations of aflatoxin B1 considering the genotype of the tester strain:

(i) The *gal* mutation makes bacteria somewhat more sensitive to certain chemicals than wild-type cells; *gal* mutants are thought to have an altered cell envelope with increased permeability [5,14].

(ii) The ΔH1 deletion, which removes *uvrB*, results in enhanced sensitivity: *uvr* mutants do not excise DNA lesions such as pyrimidine dimers and are thus more sensitive to many chemical carcinogens [3,11,19].

These two mutations, which affect the composition of the cell envelope and the mechanism of DNA repair, make the tester strain about 4-fold more sensitive than D21 which is *gal*$^+$ *uvr*$^+$ [20]. With the latter, lysogenic induction was maximum at a concentration of aflatoxin B1 of about 100 µg/ml [20] instead of 25 µg/ml in the case of SA 1512 bacteria.

Capacity of the treated cells to produce galactokinase

Treatments that induce prophage λ, i.e. that inactivate *cI*$^+$ repressor, generally have two antagonistic effects. They trigger the multiplication of the prophage and they have a toxic effect: the capacity of the treated cell to reproduce the induced phage or, in the present case, the capacity to synthetize an enzyme, may be abolished or decreased. In order to determine the capacity of the treated cells to synthetize galactokinase, we have used strain SA 1511, similar to SA 1512, except that it carries a mutant gene *cI857* which directs the synthesis of a thermolabile repressor. When a culture of strain SA 1511 is shifted from 32° to 42°, λ repressor is inactivated and the synthesis

of galactokinase is fully induced in every cell. Therefore, a reduction in the level of galactokinase activity, when the cells are pretreated with a chemical prior to the shift, indicates that the tested chemical has a deleterious effect on the cellular capacity to synthetize the enzyme.

In Fig. 3, we see that at concentrations of aflatoxin B1 higher than 10 μg/ml, the amount of galactokinase produced declined: this shows the toxic effect of aflatoxin B1 metabolites on the cells.

Synthesis of galactokinase was not induced at 32° by 25 μg/ml of aflatoxin; this is due to the fact that the λ*cI857* repressor is not inactivated at 32° whatever the inducing treatment used. The *cI857* cistron is known to carry a second mutation, *ind*, which renders the repressor insensitive to UV induction [27].

Note (Fig. 4) that the toxic effect of aflatoxin B1 metabolites prevented the cells from reaching the maximum level of galactokinase induction (170 units/ml) obtained in the case of the thermoinducible mutant SA 1511 in the absence of aflatoxin B1. However, the toxicity appeared only at concentrations of aflatoxin B1 20-fold higher than the minimal inducing concentration (0.5 μg/ml). This suggests that, while at high concentrations of aflatoxin B1 RNA and protein synthesis are strongly inhibited [21,23], DNA is the most sensitive target of aflatoxin B1 metabolites. The DNA lesions [7] induce a complex metabolic pathway [20,22] leading in our tester strain to the induction of prophage λ and, consequently, to the read-through transcription of the *galK* gene.

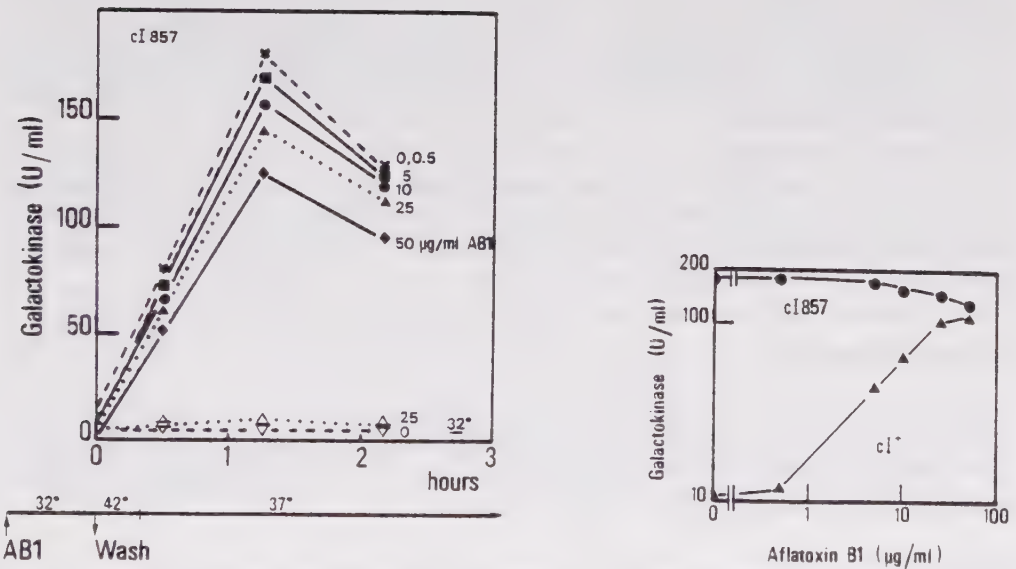

Fig. 3. Capacity to produce galactokinase after treatment with activated aflatoxin B1. Thermoinducible bacteria SA 1511 were treated as described in Materials and Methods; posttreatment incubation was at 42° for 20 min and then at 37° except for two samples (△) (▽) which were incubated at 32°.

Fig. 4. Capacity to produce galactokinase and induction of galactokinase vs. concentration of aflatoxin B1. The data were taken from Figs. 1 and 2. Values at 70 min of incubation are given.

Discussion

The correlation between the carcinogenic activity of chemical compounds and their prophage inducing activity has been recently extended [19] and the implication of this correlation has been discussed [20].

We propose here a new assay for chemical carcinogens, still based upon lysogenic induction now measured as an induced enzymatic activity. To achieve this, a strain has been constructed in which the *gal* operon is under the negative control of the prophage λ repressor. Using a potent carcinogen, aflatoxin B1, activated in vitro with enzymes from rat-liver microsomes, we have shown that the prophage λ inducing activity of a chemical may be detected within 2 h after treatment of the tester bacteria. This assay provides a simple way to test rapidly the prophage-inducing activity of potential carcinogens [20] as originally proposed by Lwoff [16].

The practicality of our method might be enhanced in many ways. For example, the sensitivity of the tester strain might be considerably increased by introducing *galU* and/or *envA* mutations, which render cells permeable to many chemicals [14]. The sensitivity of lysogenic cells could be enhanced 1000 times by the combination of *envA* and *uvrB* mutations [19]. Another improvement would be to measure an enzymatic activity that does not require radioactively labeled compounds. For example, induction of the synthesis of β-galactosidase in cells whose *lac* operon is fused to the λ*cI* gene would provide an easy test (R. Elesperu and M. Yarmolinsky, personal communication).

Recently, Smith and Oishi [26] have proposed a biochemical assay of prophage induction based upon the read-through transcription of the *trp* operon. Though based upon an approach similar to ours, the method of Smith and Oishi seems to require an additional step, not necessary in our assay, that is superinfection of the lysogenic cells with λ*ptrp* or Φ80*ptrp* phages prior to the inducing treatment.

In order to perform our assay, we have constructed a strain where a cryptic virus controls negatively a chromosomal gene; a carcinogen, by causing the inactivation of a viral repressor, turns on the expression of a chromosomal gene. Such a situation has been postulated to account for the initiation of transformation of mammalian cells [13,15].

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ASPECT QUANTITATIF DES TESTS BACTERIENS

Comment établir des doses admissibles pour chaque cancérogène chimique présent dans notre environnement ?

Pour tenter d'apporter une réponse à cette question, il est nécessaire tout d'abord de définir une "unité" qui permette de mesurer l'efficacité relative de chaque produit chimique. Une unité qui ferait référence aux rayonnements (X ou UV) présente des avantages certains puisque dans ce cas les doses admissibles sont relativement bien établies.

Quel phénomène choisir pour mesurer l'efficacité d'un produit chimique à endommager l'ADN d'une cellule ? Un système basé sur l'induction lysogénique présente deux avantages par rapport aux systèmes de mutagénèse :

a) les courbes dose-effet sont linéaires pour les doses faibles,

b) l'induction est largement indépendante de l'effet toxique d'un produit.

(Goze, A., Moreau, P. and Devoret, R. (1977), in R. Chanet (Ed.) First European Symposium on Rad-Equivalence, Commission of the European Communities, Luxembourg, pp. 251-260).

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PROPHAGE λ INDUCTION BY RADIATIONS AND CARCINOGENS ; THEIR RELATIVE EFFICIENCY

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Carcinogenic agents have been shown to be prophage inducers (1) and lysogenic induction has been used to detect potential carcinogens (2). The effect of ionizing radiations and UV-light on prophage induction has been extensively studied as well as their effects on phage and bacterial survival (3). There are some similarities between the events leading to prophage induction in bacteria and to carcinogenic transformation in eucaryotic cells (4). All these justify the use of lysogenic induction to estimate the efficiency of chemical carcinogens in terms of radiation units equivalents.

Aflatoxin B1 metabolites mimic the effect of UV-light on *E. coli* K12.

The mycotoxin aflatoxin B1 is a powerful carcinogen commonly found in peanuts and cereals (5). Like most carcinogens, it must be metabolized by mammalian microsomal enzymes to give rise to compounds active on bacteria (6). Aflatoxin B1 metabolites induce prophage λ and phage reactivation (4, 6) through the occurrence of DNA lesions that are subject to exision repair (2, 4, unpublished results). Aflatoxin B1 metabolites produce DNA damage similar to pyrimidine dimers.

Physiological functions used in order to compare the relative efficiency of carcinogens metabolites with radiations

DNA lesions due to X-rays are not repaired by the excision repair system of the bacteria (7). Therefore, we must compare the effect of irradiation or carcinogen exposure on *Uvr*$^-$ bacteria which are excision repair deficient. Similarly, phage λ survival must be measured after propagation on *Uvr*$^-$ bacteria.

1. Bacteriophage and bacterial survival

37 % Survival	X-ray dose k rads	UV fluence J/m^2	Aflatoxin B1 concentration µg/ml
Phage λ (Fig. 1)	100	8	4
E. coli K12 (Fig. 2)	7	1	10

Measurement of the survival of phage or bacteria might be a way of standardizing effects of radiations and drugs. This method, however, is not very sensitive : for example, 100 krads give 37 % phage λ survival. On the contrary lysogenic induction is a highly sensitive test.

2. Lysogenic induction of *E. coli* K12 (λ)

Proportionality between the number of induced bacteria and the X-ray dose has been demonstrated experimentally at very

low doses. The dose effect relationship is linear and, if plotted on a log scale, the representative curve is a straight line having a slope of 1 (3). At low doses the probability that two photons cross the same bacteria is very low. Thus, only one photon can induce the development of prophage λ. Prophage induction by X-rays is clearly a phenomenon without threshold, however low the dose might be. The same results are obtained with γ rays of ^{60}Co or α particles (8).

In the case of UV-light, the dose response curve plotted logarithmically is a straight line having a slope of two : this means that prophage induction results from two independent events (two-hit curve).

In order to increase the sensitivity of the lysogenic system to chemical carcinogens, we used an *E. coli* K12 strain permeable (*envA*) also deficient in DNA repair (*uvrB*). Lysogenic induction of prophage λ in this tester strain by aflatoxin B1 and benzo(a)pyrene metabolites is shown in Fig. 2. The dose response curves are approximately two hit.

Calculation of rad equivalents

As prophage induction by X-rays and by chemical carcinogens we used have not the same type of dose response curve, we have compared them at doses giving the same number of events per bacteria. The dose of X-rays giving 63 % of induced cells (15 krads) corresponds approximately to a UV fluence giving

26.4 % of induced cells (3 J/m²) : at these doses one event occurs per cell. Twenty six per cent of induced cells correspond to about 25 ng/ml of aflatoxin B1 and 1500 ng/ml of benzo(a)pyrene.

We have calculated the number of nanograms of the drug corresponding to one rad (Table 1). Aflatoxin B1 is a very efficient prophage inducer and it is also the most potent liver carcinogen as yet discovered. The differences in λ induction abilities of various aflatoxins are related to their carcinogenic potential in animals : aflatoxin G1 is a weak carcinogen compared to aflatoxin B1. Sterigmatocystin is slightly less effective than aflatoxin B1.

Calculation of pyrimidine dimers equivalents

Aflatoxin B1 metabolites affect DNA as UV-light does. 0.1 J/m² makes about 6 pyrimidine dimers per bacterial genome. In *Uvr*⁻ bacteria, 37 % cell survival correspond to a UV fluence of about 1 J/m², i.e. approximately 60 pyrimidine dimers per genome (7), and to 10 µg of aflatoxin B1 (Fig. 1). One can estimate that 1 µg of aflatoxin B1 causes lesions equivalent to about 6 pyrimidine dimers per treated cell, whereas for phage survival it is equivalent to about 12 pyrimidine dimers.

Lysogenic induction is far more sensitive : 25 ng of aflatoxin B1 have the same inducing effect as 3 J/m². From these values one can estimate that the inducing activity of

1 ng of aflatoxin B1 corresponds to about 6 pyrimidine dimers : this is 10^3 times more than values obtained with bacterial survival. This increase in sensitivity corresponds to the increase in permeability of the bacterial cell wall of the *envA* mutant and to the higher sensitivity of lysogenic induction compared to bacterial survival.

Conclusion :

As aflatoxin B1, many carcinogens mimic the biological action of UV-light on bacteria and induce excisable lesions on DNA (9, 10). The use of the notion of pyrimidine dimers equivalent seems more adapted to the case than the notion of rad equivalent, which is nevertheless useful.

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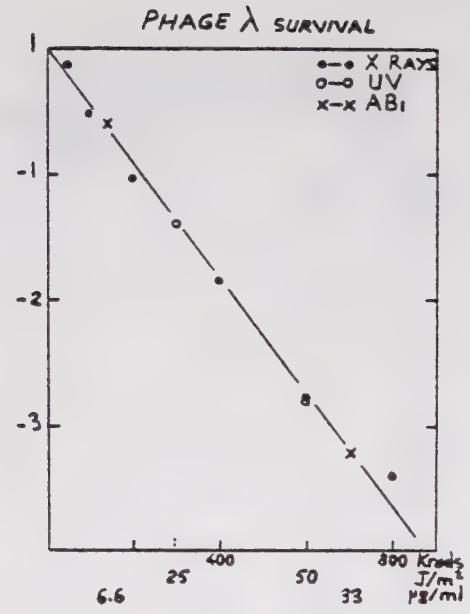
TABLE 1. Estimation of rad equivalents : number of nanograms of the chemical carcinogen corresponding to one rad.

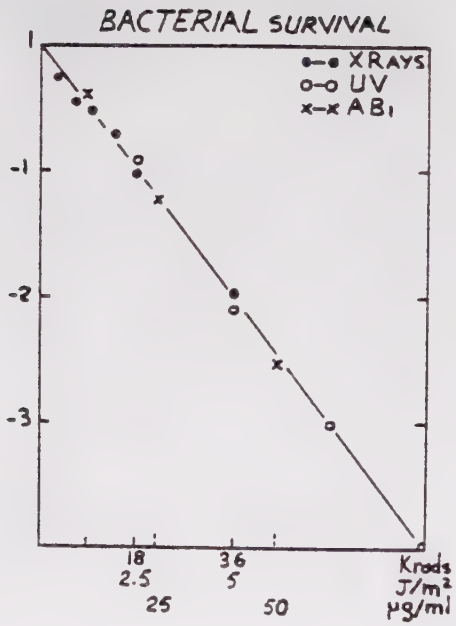
Chemical carcinogens	Values in ng calculated from :		
	λ Survival on AB1886 *uvrA6*	Bacterial survival AB1886 *uvrA6*	Prophage λ induction GY5027 *envA uvrB34* (λ)
Aflatoxin B1	0.04	1.4	0.0016
Aflatoxin G1	-	-	0.066
Sterigmatocystin	-	-	0.002
Benzo(a)pyrene	-	-	0.1

FIGURE 1.



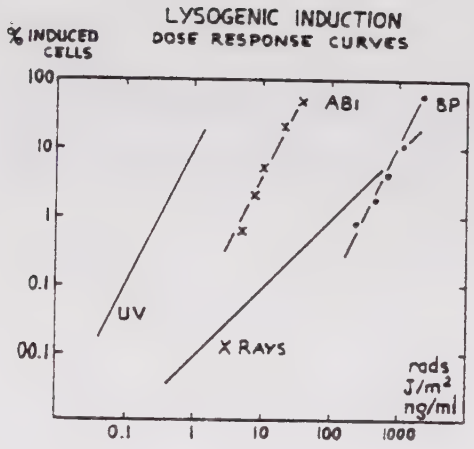
Phage λ survival has been measured on AB1886 *uvrA6* after UV irradiation or aflatoxin B1 metabolites treatment, on K12S after X-rays irradiation (3). The scales of X-ray dose, UV fluence and aflatoxin B1 concentration have been adjusted to superimpose the survival curves.

FIGURE 2.



Bacterial strains used were AB1157 for the measurement of cell survival after X-rays irradiation and AB1886, its *uvrA6* derivative, for the measurement of cell survival after UV irradiation or aflatoxin B1 metabolites treatment. As in Fig. 1, the scales of the X-axis have been adjusted to superimpose the survival curves.

FIGURE 3.



The percentage of induced cells is plotted against X-ray dose, UV fluence, aflatoxin B1 and benzo(a)pyrene concentration. Bacterial strains used were K12S (λ) for X irradiation, AB1886 *uvrA6* (λ) for UV irradiation, GY5027 *envA uvrB34* (λ) for aflatoxin B1 and benzo(a)pyrene metabolites treatment.

UTILISATION DE L'INDUCTEST POUR LA DETECTION DE SUBSTANCES ANTITUMORALES

Le développement de nouvelles substances antitumorales découle le plus souvent de "l'amélioration" de substances actives déjà connues. Peut-on aider les chimistes organiciens à orienter leur recherche dans telle ou telle direction en prédisant l'activité antitumorale que peut avoir un dérivé, et ce en utilisant un test d'induction lysogénique ? Pour apporter une première réponse à cette question, nous avons comparé les activités inductrices et antitumorales de 16 dérivés de la famille des anthracyclines.

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INDUCTION OF PROPHAGE λ BY DAUNORUBICIN AND DERIVATIVES

CORRELATION WITH ANTINEOPLASTIC ACTIVITY

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Summary

The antineoplastic drug daunorubicin and 15 other anthracyclines were tested for their ability to induce prophage λ in *Escherichia coli* K12. Prophage λ induction by daunorubicin was obtained in excision-repair deficient *uvr*$^-$ bacteria at doses about 3-fold lower than in excision-repair proficient *uvr*$^+$ cells; this suggests that some of the lesions produced in DNA by daunorubicin are subject to excision repair and may be adducts. Daunorubicin seems to be converted to active species capable of causing prophage inducing lesions in DNA by bacterial enzymes. The antineoplastic and prophage inducing potencies of the anthracyclines were compared in a blind test. These two parameters were correlated for two thirds of the compounds. Such a correlation supports the idea that the antineoplastic activity of the anthracyclines is a consequence of their capacity to damage DNA.

Daunorubicin and Adriamycin (doxorubicin) are members of the anthracycline family of antineoplastic drugs (Fig. 1). They are extensively used in cancer chemotherapy, most notably in the treatment of human leukemias, but also in the treatment of solid tumors [12]. Both daunorubicin and Adriamycin are potent mutagens in *Salmonella typhimurium* [6,27,39]. It has been proposed [12] that the antineoplastic and mutagenic activities of these compounds are

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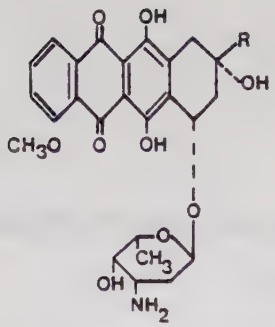

Fig. 1. Structure of some anthracycline antibiotics. Daunorubicin, R = —CO—CH_3; Adriamycin (doxorubicin), R = —CO—CH_2OH; Rubidazone (zorubicin), R = —$(CH_3)C$ = N—NH—CO—C_6H_5; daunorubicinol, R = —CHOH—CH_3.

related to their strong intercalative binding to DNA [12,36].

Since the original suggestion by Lwoff in 1953 [26], prophage λ induction has been used to screen for potential chemical carcinogens [15,20], as well as for antitumor antibiotics [7,20,34]. An improved test has been described recently [29,30]. In this newly developed inductest the tester bacteria carry a mutation, *envA* [32], that renders them more permeable than wild-type cells to many chemicals. A second mutation, *uvrB34*, can be introduced to make them deficient in excision repair of many DNA lesions [18]. Most chemicals require enzymatic activation for conversion into forms capable of damaging DNA [28], and this activation can be achieved with a metabolizing mixture [14].

Prophage induction as well as mutagenesis result from a complex series of cellular reactions usually triggered by DNA damage [11,35,43]. In general, therefore, the inductest and the Salmonella mutagenicity test [2] detect chemicals that damage DNA. Many antitumor agents bind strongly to DNA and are also DNA-damaging agents [22].

For various agents, including daunorubicin and Adriamycin, a qualitative correlation between antineoplastic and inducing * activity has been demonstrated [20,34]; this correlation has permitted the use of induction as a prescreen for the detection of new antitumor antibiotics [7].

We reasoned that for the chemical derivatives of an antitumor agent known to induce prophage λ, the inducing potencies of the derivatives could be used to predict their antineoplastic potencies in mammals. This hypothesis was tested by subjecting 16 anthracyclines to the inductest. Among the compounds studied were daunorubicin and Adriamycin as well as daunorubicinol and Rubidazone (zorubicin) (Fig. 1). 12 other anthracyclines, including *N*-(L-leucyl)-daunorubicin, were also tested; the antineoplastic potencies of these compounds were unknown to 3 of us (W.A.A., P.L.M. and R.D.). The antineoplastic activities were measured at Rhône-Poulenc (R.M.), and, for the 12 compounds in the blind series, the results were revealed to the Gif group only after the inducing potencies had been determined.

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* In this paper the terms "induction", "inductest" and "inducing" refer always to the induction of prophage λ in cells of *Escherichia coli* K12.

We present here an analysis of the inducing properties of the 16 anthracyclines and the correlation between their antineoplastic and inducing potencies.

Materials and methods

Bacterial strains

The *E. coli* tester strains [29,30] and their relevant genetic markers are GY 5022 *envA uvr*$^+$ (λ), which is the *lam*$^+$ parent of GY 5023 [30], and GY 5027 *envA uvrB34* (λ); they are both resistant to 200 μg/ml of streptomycin. Phage λ was detected as plaques on a lawn of GY 4015 indicator bacteria, which are resistant to 15 μg/ml of ampicillin. The permeability of the *envA* tester bacteria to gentian violet as well as the sensitivity of the *uvrB34* tester cells to ultraviolet light was checked routinely as described by Ames et al. [2].

Media and culture conditions

LB: Difco yeast extract, 5 g; Difco tryptone, 10 g; NaCl, 10 g; water, 1 l. LBE: LB supplemented with 0.2% glucose and 20% medium E of Vogel and Bonner. LBES: LBE, 1 l; Biomar agar, 12 g; supplmented with 200 μg/ml of streptocycin. GT-amp: peptone; 8 g; Difco tryptone, 5 g; NaCl, 5 g; Biomar agar, 12 g; water, 1 l; supplemented with 15 μg/ml of D-ampicillin. Soft agar: Difco agar, 7.5 g; water, 1 l.

For all experiments fresh overnight cultures of λ lysogens in LBE medium were diluted 1 : 100 into fresh LBE medium, grown with aeration at 37° C to about 6×10^7 viable cells per ml and used promptly.

Chemicals

NADP, aflatoxin B1 and glucose-6-phosphate were from Sigma. Aroclor 1254 was a generous gift of Monsanto Chemicals. Anthracyclines were synthesized in the Rhône-Poulenc Industries Research Center and were used as the hydrochloride salts; solutions were freshly prepared at 2 mg/ml in sterile water on the day of the induction experiment and protected from light. Aflatoxin B1 was dissolved in dimethylsulfoxide and stored at −20° C.

Prophage λ induction

Induction experiments were performed in liquid medium essentially as described previously for inductest III [29,30], except that the reaction volumes were 0.1 ml. All results presented in a given figure or table were obtained on a single day with the same batch of cells for each strain used.

The number of viable cells of *envA* tester bacteria used in each experiment was determined as described by Moreau et al [29] except that LBES agar plates were used. These plates allowed better growth of *envA* cells than did LB agar plates supplemented with calcium [31].

The metabolizing mixture [30] normally consisted of a rat-liver homogenate (a 9000 *g* supernatant designated S9) plus cofactors (NADP, $MgCl_2$ and glucose 6-phosphate) in phosphate buffer. In some experiments S9 or cofactors were omitted. The S9 was prepared in the laboratory of Dr. F. Zajdela from rats induced with Aroclor 1254 [2]. 3 μl of S9 were used per 0.1-ml assay; for each of 3 different S9 extracts used, this amount gave maximal activation of aflatoxin B1 and daunorubicin.

TABLE 1

COMPARISON OF ANTINEOPLASTIC ACTIVITY AND MINIMAL INDUCING DOSE OF 16 ANTHRACYCLINES

Rhône-Poulenc code number	Name	Gif code letter(s)	MTD [a] (mg/kg)	Antineoplastic activity [b] at						Minimal inducing dose [c] (ng/assay)
				4 MTD	2 MTD	MTD	MTD/2	MTD/4	MTD/10	
13 057	daunorubicin	DNR	0.75	56	188	185	180	154	103	13
25 253	Adriamycin	ADR	0.75	196	199	188	175	140	111	35
22 050	Rubidazone	C	3.00	—	182	186	164	152	119	70
20 132	*N*-(L-leucyl)-daunorubicin	D	1.25	183	178	165	168	122	—	1500
33 921		E	1.25	125	200	196	189	176	125	24
20 798	daunorubicinol	F	3.00	—	189	175	170	149	107	140
30 449		G	0.20	—	80	183	151	110	—	12
33 543		H	5.00	—	200	174	184	123	98	42
33 091		I	5.00	—	—	97	99	—	—	800
33 379		J	15.00	—	101	99	103	101	101	200
34 230		K	37.50	—	146	176	165	125	104	200
35 263		L	1.25	—	—	123	122	113	—	70
34 835		M	5.00	—	129	136	108	97	—	17
35 407		N	50.00	—	—	194	173	144	120	210
38 954		O	5.00	—	—	160	107	107	113	10
38 422		P	12.50	—	—	100	100	100	100	25

[a] MTD is the maximum tolerated dose following intraperitoneal injection. It is the maximum dose causing neither mortality nor weight loss in the treated mice.

[b] The antineoplastic activity is expressed as the percent survival time of the treated mice as compared to the control mice and was calculated as described in Materials and Methods.

[c] The minimal inducing dose is expressed in ng per 0.1 ml assay and was determined as described in Materials and Methods. Assays were performed in the presence of S9 as described in the legend to Fig. 2. Maximum induction for all compounds was equal to or greater than 60 percent except for compound D (*N*-(L-leucyl)-daunorubicin) which gave 20% induction at the highest dose tested (5 μg).

Inducing potency of a chemical

The induction curve obtained by plotting the number of λ infective centers per assay as a function of the dose of an inducing chemical is typically composed of 4 parts (see ref. 30 and Fig. 2). At low doses no increase over the background of spontaneously produced infective centers can be detected (part 1). At the minimal inducing dose the ascending portion of the curve (part 2) begins. At still higher doses the curve reaches a maximum; for some chemicals this maximum is maintained over a wide range in the form of a plateau (part 3). At toxic doses the curve falls (part 4), the capacity of the cells to reproduce the induced prophage being affected. The inverse of the minimal inducing dose is taken as an index of the inducing potency of a chemical.

Antineoplastic activity

The antileukemic activity of the anthracyclines was measured using the L1210 leukemia. Mice ($B_6D_2F_1$ male or female) were injected intraperitoneally with 10^3 L1210 cells on day 0. The anthracyclines were administered by intraperitoneal injection on days 0, 1, 2, 3 and 4. Animals were observed for 30 days, which was twice the average survival time of controls not treated with an anthracycline.

The antineoplastic activity of a drug at a given dose is expressed as the following percentage: (mean survival time of the anthracycline treated animals)/(mean survival time of control animals) × 100. Values equal to or greater than 125 signify substantial activity.

Antineoplastic potency of a chemical

The antineoplastic potency was obtained by dividing the antineoplastic activity at the maximum tolerated dose by the numerical value of that dose. It is a measure of the specific antineoplastic activity. For example, anthracyclines G and N displayed similar antineoplatic activities (183 and 194 resp.; see Table 1) for maximum tolerated doses that differed by a 250-fold factor; thus their antineoplastic potencies differ by nearly 250-fold as well.

Results

Anthracyclines induce prophage λ

It has been reported that daunorubicin and Adriamycin are mutagens [6,27, 39] and induce prophage λ [7,34] without enzymatic activation. We wished to compare the inducing and antineoplastic potencies of daunorubicin and its derivatives. Antineoplastic action of a chemical may be a consequence of its metabolization in vivo. For our comparative study, therefore, the induction experiments were performed using a metabolizing mixture.

We found daunorubicin, the parent compound of the anthracyclines studied, to be an effective inducer of prophage λ with and without enzymatic activation. Without S9, the minimal inducing dose for the excision-deficient strain, GY 5027, was about 150 ng (Fig. 2). When S9 was present the minimal inducing dose was as much as 8 times lower (Fig. 2), although the activation factor was somewhat variable (compare Figs. 2 and 3). Aflatoxin B1, which has an absolute requirement for enzymatic activation before it will induce prophage λ

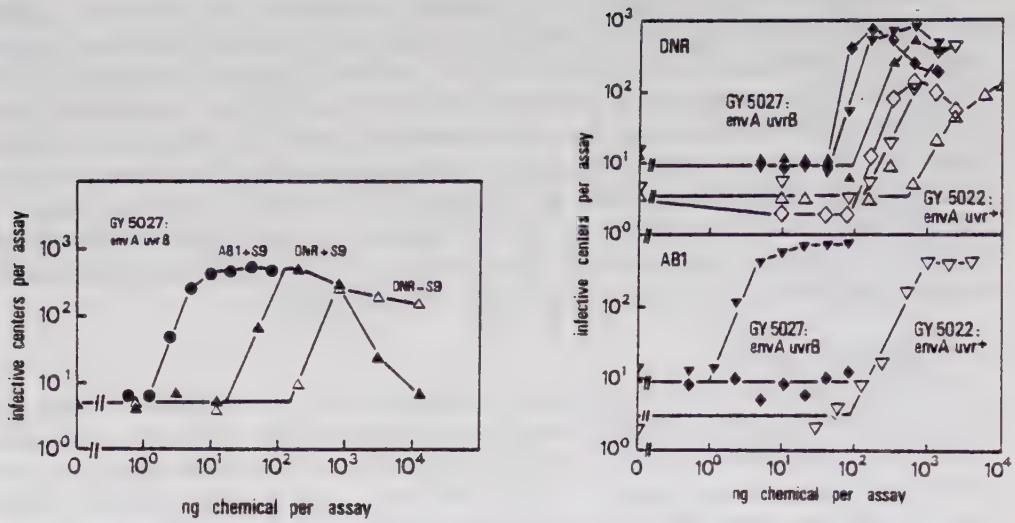

Fig. 2. Induction of prophage λ by daunorubicin (DNR) and aflatoxin B1 (AB1). 2 mixtures [30] were prepared. One (closed symbols) contained GY 5027 tester cells, cofactors and 30 μl of S9 per ml, and the other (open symbols) contained tester cells and cofactors but no S9. Aliquots of 0.1 ml of the mixtures were distributed to tubes containing the product to be tested in 5 μl of distilled water for daunorubicin (▲, △) and in 5 μl of dimethylsulfoxide for aflatoxin B1 (●). After 25 min of incubation at 37° C without shaking, the contents of each tube were poured onto GT-amp plates with 3 ml of soft agar containing 0.2 ml of an overnight culture of GY 4015 indicator bacteria. Plates were incubated overnight at 37° C following which the infective centers were counted. The number of viable cells per 0.1-ml assay was 4.2×10^2.

Fig. 3. S9-dependent activation of daunorubicin does not require exogenous cofactors and daunorubicin induces prophage λ at low soses in excision-deficient bacteria. The number of viable cells per 0.1-ml assay was 9.0×10^2 for the excision-repair deficient strain, GY 5027 *envA uvrB34* (closed symbols) and 6.7×10^2 for the excision repair proficient strain, GY 5022 *envA uvr*$^+$ (open symbols). *Upper panel*: Prophage λ induction by daunorubicin (DNR) was assayed as described in the legend to Fig. 2. Excision-repair deficient and excision-repair proficient cells were tested, each in the presence (▼, ▽) and in the absence (▲, △) of S9. Assays were also performed in the presence of S9 but in the absence (◆, ◇) of cofactors ($MgCl_2$, NADP and glucose 6-phosphate). *Lower panel*: Prophage λ induction by aflatoxin B1 (AB1) was assayed in the presence of S9 (▼, ▽) as described in the legend to Fig. 2. For the excision-deficient strain only, cofactors were omitted from one series of tubes (◆).

[17], was used as a control; the minimal inducing dose was approx. 1 ng (Fig. 2; see also ref. 29).

As with aflatoxin B1, daunorubicin induced prophage λ with high efficiency in that all or nearly all of the treated cells formed infective centers. A toxic effect was evident at doses of daunorubicin close to those required for maximal induction. This was displayed by a reduction of the plateau region of the induction curve of daunorubicin by comparison with that of aflatoxin B1 (Fig. 2).

Adriamycin, daunorubicinol, Rubidazone and *N*-(L-leucyl)daunorubicin all induced prophage λ when assayed with S9, and their potencies varied over a 50-fold range (Table 1). Adriamycin, daunorubicinol and Rubidazone also induced prophage λ in the absence of S9, but *N*-(L-leucyl)daunorubicin, at doses up to 10 μg per assay, was inactive without S9 (data not shown).

Exogenous cofactors are not required for the enzymatic activation of daunorubicin

The oxidative activation of carcinogens such as aflatoxin B1 is catalyzed by a microsomal enzyme system and requires molecular oxygen and NADPH(reviewed in ref. 28). When the cofactors necessary for NADPH generation were omitted from the reaction mixture, aflatoxin B1 failed to induce prophage λ (Fig. 3; see also ref. 17). The enzymatic activation of daunorubicin is clearly different from that of aflatoxin B1. The enhancement of daunorubicin activity by S9 was at least as good, if not better, when cofactors were omitted as when they were present; the same result was obtained with excision-repair proficient and excision-repair deficient tester bacteria (Fig. 3).

Prophage λ induction by daunorubicin in occurs in an excision-repair deficient mutant at lower doses that in the parent

Correndonuclease II, the product of genes *uvrA*, *uvrB* and *uvrC* in *E. coli* [38], is required for the early steps in the excision repair of pyrimidine dimers produced in DNA by far ultraviolet irradiation (reviewed in ref. 18); it is also involved in the removal of interstrand cross-links from DNA [9]. Since excision-repair deficient bacteria display an increased sensitivity to the mutagenic and lethal effects of many chemicals [1,21], it seems that the bulky DNA lesions produced by these agents are subject to excision repair. We infer that whenever prophage λ induction by a given agent occurs in excision-repair deficient bacteria at lower doses than in their *uvr*$^+$ parents, then at least some of the DNA damage produced by the agent involves the formation of excisable lesions. Given that aflatoxin B1 [29,30] (see also Fig. 3) and *N*-hydroxy-2-aminofluorene (Anderson, Devoret and Saint-Ruf, to be published) induce prophage λ in the excision repair-deficient strain GY 5027 at doses that are up

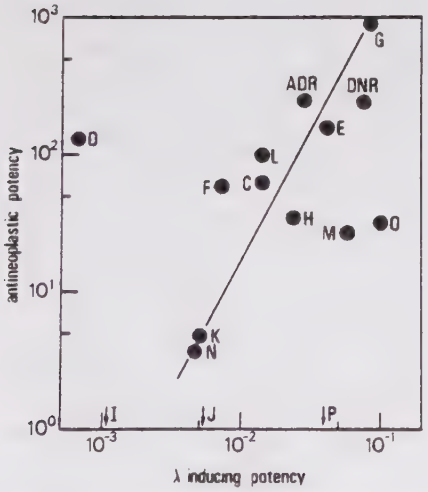

Fig. 4. Relation between antineoplastic and prophage inducing potency for 16 anthracyclines. The antineoplastic and inducing potencies were computed as described in Materials and Methods from the data of Table 1. The inducing potencies of the 3 compounds without detectable antineoplastic activity (I, J and P) are indicated by arrows on the abscissa.

to 50-fold lower than in the correpsonding *uvr*$^+$ strain GY 5022, we conclude that the inducing lesions produced by these chemicals are excisable.

Daunorubicin induced prophage λ in excision-repair deficient cells at doses that were about 3-fold lower than those required to induce the prophage in excision-repair proficient cells and this difference in sensitivity was not changed by the presence of S9 (Fig. 3).

Comparison of antineoplastic and inducing activities of the anthracyclines

In addition to the 5 compounds already discussed, we established induction curves (not shown) for 11 other metabolized anthracyclines in the excision-repair deficient strain GY 5027. From these curves we determined the minimal inducing dose for each compound. The antineoplastic and inducing activities of the 16 anthracyclines are summarized in Table 1.

For 12 compounds we find that the relationship between the antineoplastic and inducing potencies corresponds to a straight line on a log—log plot (Fig. 4). Among these compounds, then, the more potent antineoplastics are the more potent inducers. Of the 4 compounds remaining, 3 (I, J, and P) induce prophage λ but have no detectable antineoplastic activity and one (*N*-(L-leucyl)-daunorubicin) is a potent antitumor agent but a very poor inducer.

Discussion

We have shown that daunorubicin and 15 of its derivatives induce prophage λ. When the assays are performed in the presence of S9, the inducing potency varies over a 150-fold range (Table 1).

Agents that inhibit DNA replication in *E. coli* trigger the expression of a series of cellular functions which in particular lead to prophage λ induction [11,35,43]. Most chemical inducers of prophage λ can damage DNA either by forming a covalent adduct (e.g. aflatoxin B1, ref. 28) or by causing base damage or removal that can lead to DNA breakage (e.g. bleomycin, ref. 24).

Anthracyclines bind very strongly to DNA in vivo and in vitro [12,36]. Furthermore, daunorubicin and Adriamycin inhibit *E. coli* DNA polymerase I and other DNA polymerases in vitro [13,44]. It is possible that tight binding of anthracyclines to DNA is sufficient in itself to inhibit DNA replication and to cause prophage induction. However, daunorubicin causes chromosome fragmentation in mammalian cells [12] and anthracyclines cause strand breaks in intracellular mammalian DNA [8,37]. We think it more likely, therefore, that DNA damage caused by the anthracyclines is responsible for prophage λ induction in *E. coli*. In this regard, Lown et al. [25] have recently reported that daunorubicin and Adriamycin, after reduction with $NaBH_4$, can cause damage leading to single-strand breaks in DNA in a reaction involving free-radical intermediates. A free-radical mechanism has also been invoked to account for the DNA damage giving rise to strand breaks caused by streptonigrin [10], mitomycin C [23], neocarzinostatin [40] and bleomycin [24]. Note that these antineoplastic agents can produce the same kind of DNA damage as the anthracyclines and that they are all effective inducers of prophage λ [20].

If daunorubicin damages DNA by a free-radical mechanism, tight binding of the molecule to DNA would undoubtedly be important for activity. Free radi-

cals generated far from DNA would be more likely to react with cellular constituents other than DNA, as Bachur et al. [5] have also noted. The 3′-amino group has been implicated in the binding of anthracyclines to DNA [33]. Thus, the low inducing potency of *N*-(L-leucyl)daunorubicin (Table 1) and the inactivity of 3′-epi-daunorubicin [3] is presumably a consequence of a low affinity for DNA of these analogues. Similar considerations could explain the observation that *N*-methyldaunorubicin and *N,N*-dimethyldaunorubicin have respectively little and no activity in the Salmonella mutagenicity test [42].

Prophage λ induction by daunorubicin occurs in excision-repair deficient *uvr*$^-$ bacteria at some 3-fold lower doses than in excision-repair proficient *uvr*$^+$ bacteria (Fig. 3). There do exist agents, such as neocarzinostatin and bleomycin, that induce prophage λ at the same dose in *uvr*$^-$ and in *uvr*$^+$ bacteria (Anderson, Moreau and Devoret, unpublished data). Therefore we argue that at least one kind of inducing lesion caused by daunorubicin is excisable. This lesion may be a hitherto unrecognized covalent adduct of DNA and daunorubicin formed in vivo, although it is also conceivable that the binding of daunorubicin to DNA is so tight that the complex is treated like a covalent adduct by correndonuclease II.

The 3-fold sensitization to daunorubicin, compared with the 50-fold sensitization to aflatoxin B1 (Fig. 3), may mean that daunorubicin causes prophage-inducing lesions that are only inefficiently excised by correndonuclease II or that it produces both excisable and non-excisable inducing lesions. It may be, in fact, that antineoplastic agents like daunorubicin cause mainly non-excisable DNA damage and that carcinogens such as aflatoxin B1 and *N*-hydroxy-2-aminofluorene produce mainly DNA damage that can be efficiently repaired by excision.

In order for free radicals to be generated from anthracyclines, the quinone ring must be reduced [4,5,16,25]. Pyridine nucleotide-linked reductions of this type can be carried out by bacterial enzymes, the most notable example being the reductive activation of mitomycin C [41]. We suggest, therefore, that anthracyclines are activated by a bacterial enzyme. This model implies that the same reactive species, probably a semiquinone derivative [4,5], is responsible for producing the prophage-inducing lesions with and without S9; the effect of S9 would be to lower the dose of daunorubicin necessary for the formation or the delivery to the DNA of the reactive species. Note too that S9 enhances the inducing activity of daunorubicin to a similar extent in *uvr*$^+$ and in *uvr*$^-$ bacteria (Fig. 3). Thus, S9 does not change appreciably the ratio between excised and non-excised inducing lesions, again suggesting that the presence of S9 in the assay mixtures does not lead to the formation of any new reactive species.

The possibility that anthracyclines must be enzymatically reduced in order to exert their biological activity has not been widely recognized. Only recently has evidence been obtained from in vitro studies indicating that enzymatic activation of these compounds leading to the formation of free-radical intermediates can take place [4,16,19].

We have compared the potencies of 16 anthracycline antibiotics in two vastly different biological assays: induction of prophage λ and antineoplastic activity against a mouse leukemia (Fig. 4). The end points of both assays require a complex series of reactions involving such factors as the transport of

the chemicals to their sensitive target and possible enzymatic activation; competing reactions leading to toxic effects and enzymatic inactivation also exist. For example, anthracycline P may be enzymatically inactivated in the animal or it be otherwise prevented from reaching its sensitive target; it is also possible that *N*-(L-leucyl)daunorubicin is deacylated prior to exerting its antineoplastic action.

Given all the complicating factors, the correlation seen in Fig. 4 between antineoplastic and inducing potency is striking. For roughly two-thirds of the compounds tested, the antineoplastic potency could have been predicted from the inducing potency. Such a correlation supports the notion that the antineoplastic activity of the anthracyclines is a consequence of their capacity to cause DNA damage. Lack of a perfect correlation may be a consequence of secondary factors unrelated to a primary effect of the anthracyclines on DNA.

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C O N C L U S I O N

Utilisation des tests bactériens pour la détection des cancérogènes et antitumoraux potentiels.

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Il est aujourd'hui reconnu que de nombreux cancers sont dus au milieu ambiant, les produits chimiques présents dans notre environnement étant plus particulièrement mis en cause. L'identification des substances potentiellement cancérogènes pour l'homme peut donc représenter un facteur important dans la lutte contre le cancer.

Les tests de cancérogénèse utilisant des animaux sont beaucoup trop longs et trop coûteux pour pouvoir être appliqués aux milliers de produits aujourd'hui disponibles et aux centaines de nouveaux produits qui font leur apparition chaque année. De nombreux tests, dits "tests courts" ont été proposés pour évaluer à court, et surtout à long terme, le danger potentiel de ces produits. Quel(s) test(s) choisir ?

Pour répondre à cette question, il faudrait tout d'abord savoir pourquoi une cellule va échapper aux contrôles qui règlent normalement la croissance des tissus, ou tout au moins, il faudrait savoir comment agissent les cancérogènes

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chimiques au niveau moléculaire. Bien que l'on ne possède aucune preuve définitive, il est généralement admis que l'événement inducteur de la cancérogénèse est une altération du matériel héréditaire, l'ADN. Détecter des cancérogènes potentiels peut donc se ramener à reconnaître des agents qui causent des dommages à l'ADN.

Les microorganismes, les bactéries en particulier, restent le matériel le plus simple et le mieux connu pour étudier les dommages que peut subir l'ADN d'une cellule.

Quelles sont les manifestations biologiques d'une altération de l'ADN chez des bactéries ? Les données récentes peuvent être résumées de la façon suivante (Fig. 1) : les lésions de l'ADN induites par les radiations ou les cancérogènes chimiques semblent être soumises aux mêmes systèmes de réparation (le système de réparation par excision des bases ou nucléotides endommagés étant le système le mieux connu au niveau moléculaire). Ces systèmes de réparation sont fidèles mais leur efficacité n'est pas totale. Les lésions résiduelles de l'ADN seraient létales si de nouvelles fonctions cellulaires n'étaient induites de façon transitoire ("fonctions SOS"). Ceci se manifeste en particulier 1) par une augmentation du nombre de mutants parmi les cellules survivantes : il y a induction d'un nouveau système de réparation infidèle ou une modification du complexe de réplication diminuant sa fidélité ("réparation SOS"). Toutes les mutations induites par les UV seraient dues à l'expression de ce nouveau processus ; 2) quand les bactéries sont lysogènes, il

y a dérépression de provirus (ou prophages) qui étaient maintenus à l'état latent. Ce dernier phénomène, l'induction lysogénique, conduit à la lyse de la cellule hôte et à la libération d'une centaine de phages par cellule induite.

FIGURE 1

Fonctions cellulaires induites par les cancérogènes.

(a) La protéine RecA contrôle négativement sa propre synthèse avec la protéine LexA. Nous avons suggéré que le complexe RecA - LexA inhibe la transcription du gène recA : il joue le rôle d'un répresseur. Après traitement des bactéries par des agents qui causent des altérations de l'ADN, le complexe RecA - LexA serait trappé par les lésions de l'ADN (la protéine RecA a beaucoup d'affinité pour les régions de l'ADN en simple brin). La dérépression du gène recA se manifeste par une synthèse rapide et importante de la protéine RecA.

Récemment, le groupe de B. Ames, à Berkeley (Université de Californie), a montré que pour plus de 300 produits, il existait une bonne corrélation entre activité cancérogène chez l'animal et activité mutagène chez des bactéries. Le test de mutagénèse est basé sur la réversion de mutations his^- chez des bactéries Salmonella typhimurium. Les mutants his^- sont incapables de se développer si on ne leur fournit pas un acide aminé essentiel, l'histidine. La sensibilité des

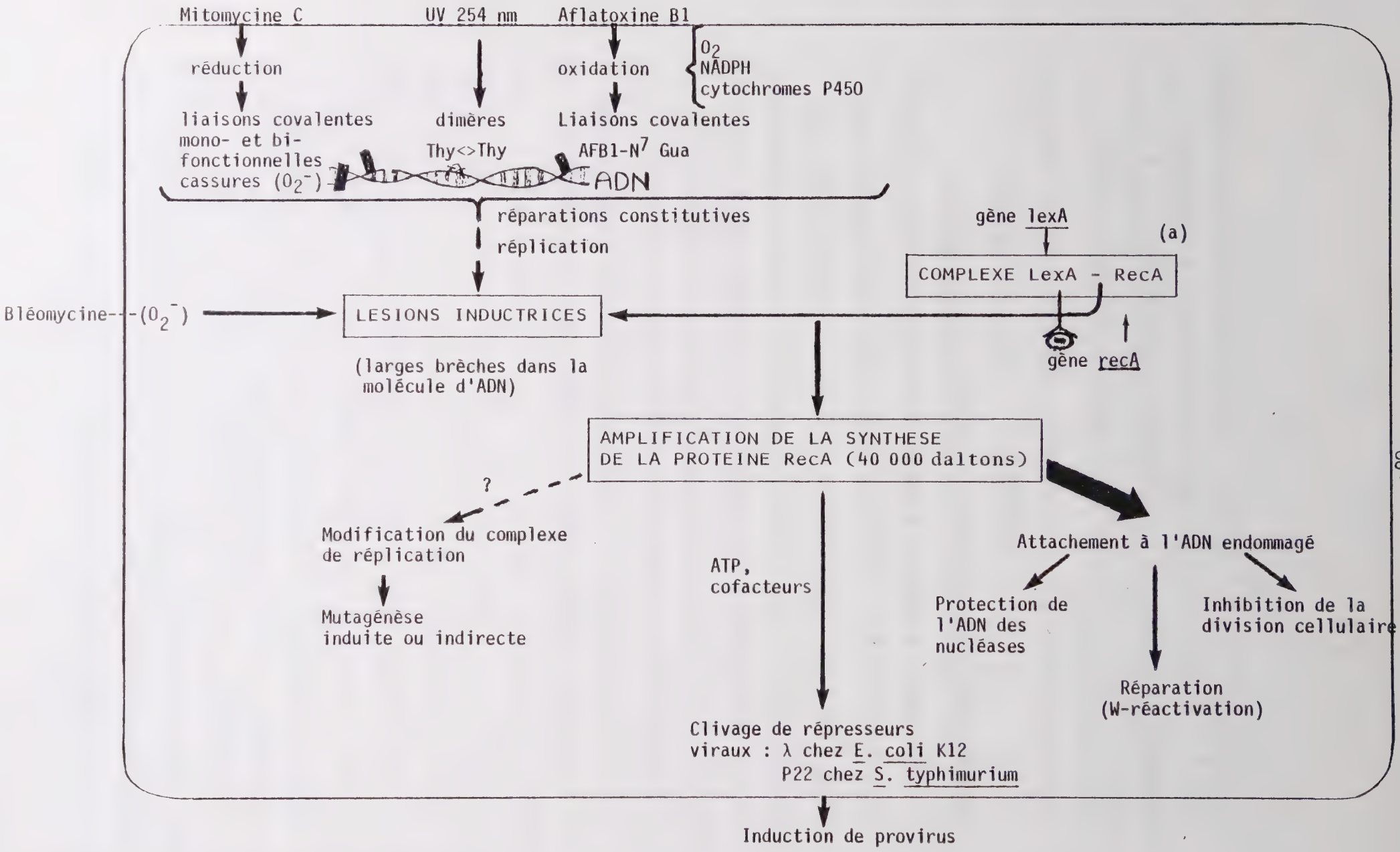


Figure 1 : Fonctions cellulaires induites par les cancérogènes.

bactéries aux produits chimiques a été accrue : 1) par une augmentation de leur perméabilité membranaire (mutation lps) ; 2) par une inhibition des systèmes de réparation de l'ADN par excision (mutation uvr). La fréquence d'induction des mutations (his$^-$ → His$^+$) est généralement augmentée par l'introduction dans les bactéries (TA98 et TA100) d'un plasmide (pKM101) codant pour la résistance à un antibiotique (l'ampicilline), et codant surtout pour de nouveaux systèmes de réparation mutagènes différents des systèmes inductibles SOS. D'autre part, la métabolisation qui subit in vivo la plupart des cancérogènes chimiques peut être reproduite par un système d'activation composé généralement d'un homogénat de foie de rat (riche en enzymes d'activation) et de NADPH.

Pratiquement, le test est réalisé de la façon suivante : 0,1 ml d'une culture de nuit de bactéries his$^-$ (10^8 cellules), 0,6 ml du mélange d'activation (ou de tampon phosphate) et une quantité déterminée du produit étudié sont étalés avec 2 ml de gélose molle sur une boîte de milieu synthétique. Chaque révertant His$^+$ donnera naissance à une colonie (10^6 cellules) visible à l'œil nu après 2 jours d'incubation à 37°C. Le nombre de révertants induits par mole de produit fournit une mesure de son activité mutagène.

Bien que très simple à réaliser, ce test ne permet pas de distinguer entre les produits qui sont des mutagènes "directs" et ceux qui sont des mutagènes "indirects". Or des agents alkylants comme l'éthyl méthanesulfonate, des analogues de base comme la 2-aminopurine, qui sont des mutagènes directs (ils provoquent de mauvais appariements des bases

au cours de la réplication de l'ADN mais ils n'induisent pas les fonctions SOS) sont peu ou pas cancérogènes.

Au contraire de la mutagénèse, l'induction lysogénique est essentiellement un phénomène indirect. Chez des bactéries lysogènes Escherichia coli K12 (λ), le prophage λ est maintenu à l'état latent par un répresseur, une protéine, qui empêche la transcription des fonctions virales. Après traitement des bactéries par des agents qui altèrent la structure de l'ADN (rayons X, UV, mitomycine C, métabolites de l'aflatoxine B1), le répresseur du phage λ est clivé par une forme activée et minoritaire de la protéine RecA (RecA protéase) (Fig. 1).

Nous avons construit une nouvelle souche bactérienne qui permet de détecter les cancérogènes chimiques par leur activité inductrice du prophage λ. Cette souche lysogène pour le phage λ est perméable aux produits chimiques (mutations envA ou lps) et déficiente pour les systèmes de réparation par excision (mutation uvrB).

En présence d'un système d'activation, la totalité d'une population bactérienne est induite par l'aflatoxine B1, une mycotoxine cancérogène, à une concentration finale de 100 µg/ml si les bactéries sont de type sauvage (env^+ uvr^+), 5 µg/ml si les bactéries portent la mutation envA, et 0,1 µg/ml si les bactéries portent les deux mutations envA et uvrB.

Pratiquement, le test est réalisé selon le protocole général suivant : 1000 bactéries lysogènes, en phase exponentielle de croissance, sont mélangées avec une quantité dé-

terminée du produit étudié (et le mélange d'activation si nécessaire) puis sont étalées sur des boîtes de milieu riche avec 2 ml de gélose molle contenant 10^8 bactéries sensibles au phage λ (bactéries indicatrices). Chaque bactérie lysogène induite donnera naissance à une plage de lyse après une nuit d'incubation à 37°C.

Pour différentes familles de composés (aflatoxines, benzopyrènes, benz(a)anthracènes), nous avons obtenu, après métabolisation, une bonne corrélation entre activité cancérogène chez l'animal et activité inductrice de λ.

Depuis de nombreuses années, des tests d'induction du prophage λ ont été utilisés (notamment par les laboratoires Bristol) pour découvrir de nouvelles substances antitumorales. Pour différents dérivés de la daunorubicine et de l'adriamycine, des antitumoraux de la famille des anthracyclines, nous avons montré que, outre une corrélation qualitative, une bonne corrélation quantitative pouvait être obtenue entre pouvoir antitumoral chez l'animal et pouvoir inducteur de λ (Fig. 2).

FIGURE 2

Relation entre le pouvoir antitumoral chez la souris et le pouvoir inducteur de λ pour 16 anthracyclines.

DNR = daunorubicine ; ADR : adriamycine ; C = rubidazone ; D = N(L-leucyl)daunorubicine ; F = daunorubicinole.

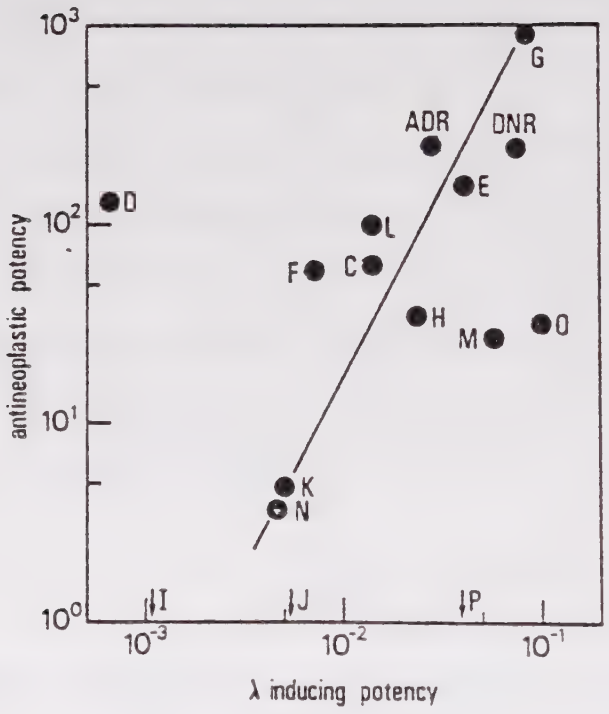

10^3
10^2
10^1
10^0
antineoplastic potency
G
ADR
DNR
E
D
L
F
C
H
M
O
K
N
I
J
P
10^{-3}
10^{-2}
10^{-1}
λ inducing potency

FIGURE 2

Quand pour un produit apparaît une divergence entre activité mutagène (chez des souches S. typhimurium) et activité inductrice de λ, il est intéressant de pouvoir comparer ces deux activités dans les mêmes conditions expérimentales. C'est pourquoi nous avons construit une nouvelle souche bactérienne qui nous permet de mesurer ces deux phénomènes simultanément. Nous avons lysogénisé la souche envA uvrB par un phage λ mutant (λ v2 v3) : il suffit d'un seul événement mutationnel (une mutation du type v1) pour inactiver l'opérateur sur lequel se fixe normalement le répresseur du phage λ. Le phage devient alors virulent ; contrairement au phage original, dit tempéré, un phage virulent peut seul se développer sur des bactéries indicatrices lysogènes.

Nous avons ainsi vérifié que l'éthyl méthanesulfonate est mutagène et non inducteur de λ, alors que certains antitumoraux (la néocarzinostatine, la bléomycine, des sels de platine...), qui sont des inducteurs puissants, sont peu ou pas mutagènes. Le cas de la bléomycine, un antitumoral utilisé cliniquement, est particulièrement démonstratif : la bléomycine induit le développement du prophage λ, le nombre de mutants λ vir est multiplié par 10, pourtant la bléomycine ne présente aucune activité mutagène sur les différentes souches Salmonella typhimurium his$^-$. De plus, le fait que les bactéries qui ne peuvent pas exciser les lésions de leur ADN (uvr$^-$) ne sont pas sensibilisées à l'action inductrice de la bléomycine suggère que les lésions de l'ADN qui sont induites sont d'un type différent de celles qui sont induites par les cancérogènes majeurs comme l'aflatoxine B1. Certaines

lésions causées à l'ADN par la bléomycine (probablement des cassures) seraient létales ; les systèmes de réparation induits (SOS) ne pourraient pas les éliminer. Cet exemple attire l'attention sur le fait que l'activité cytotoxique d'un produit peut masquer son activité mutagène. Ceci est également vrai dans le cas d'autres drogues antitumorales, la mitomycine C (si les souches portent la mutation uvr$^-$) et l'actinomycine D (tableau 1).

En conclusion, il apparaît que le test d'induction du prophage λ fournit un complément important aux tests de mutagénèse pour le dépistage d'agents qui causent des lésions de l'ADN et qui sont donc des cancérogènes ou des antitumoraux potentiels.

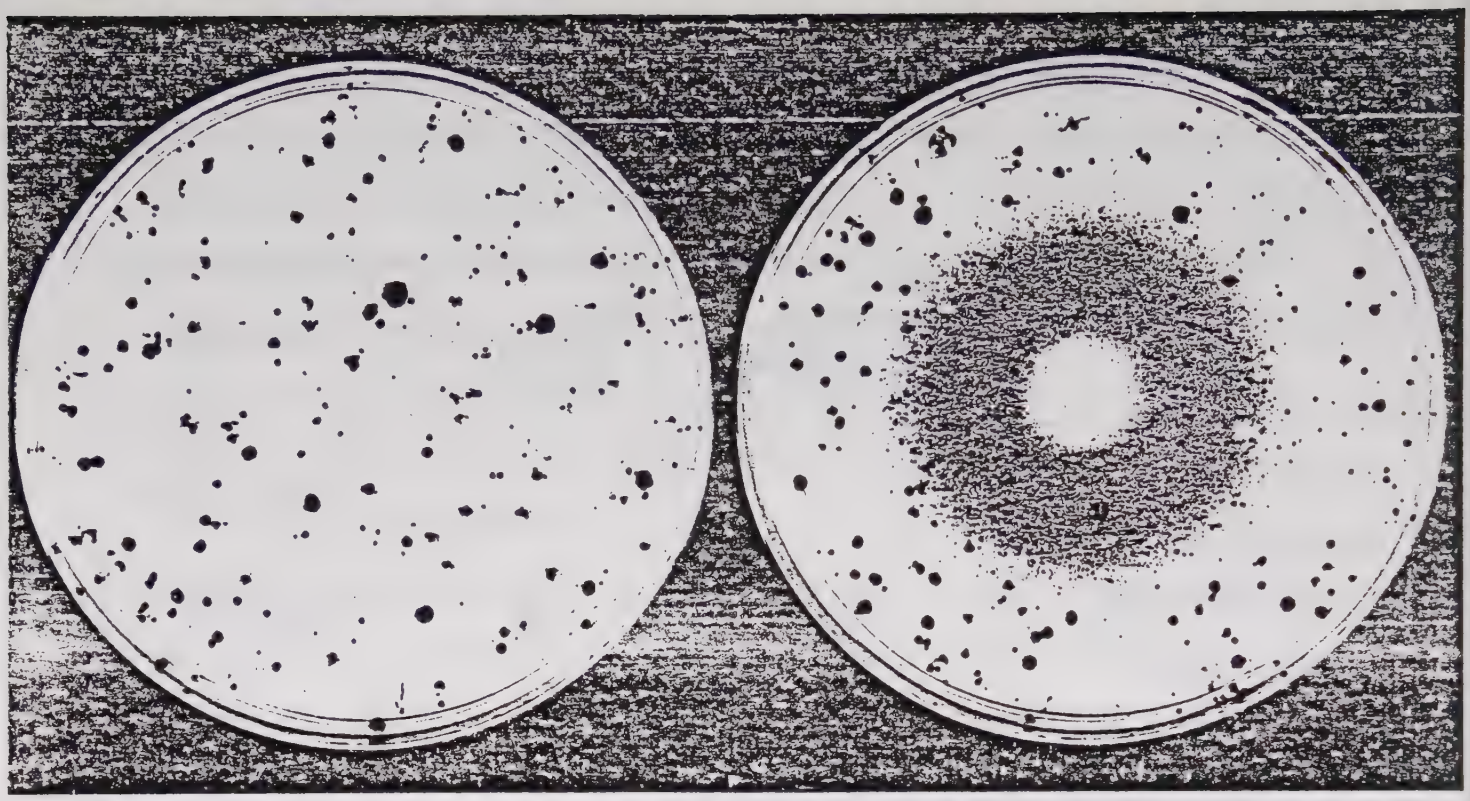
Nous avons émis l'hypothèse que chez les cellules eucaryotes, l'induction de nouvelles fonctions (SOS) par les lésions de l'ADN pourrait être responsable de l'initiation de la cancérogénèse. Des expériences récentes sur des cellules de mammifères confirment cette hypothèse.

Ouvrages de base :

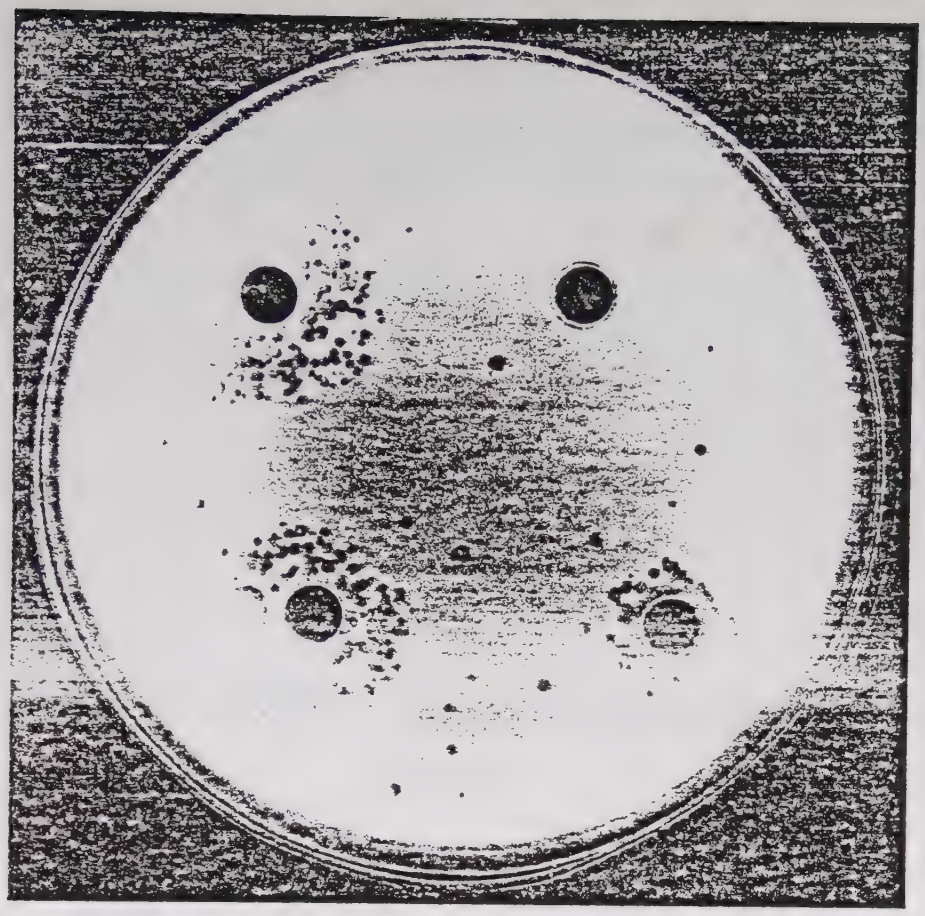
- Origins of Human Cancer. Cold Spring Harbor Conferences on Cell Proliferation (1977) vol. 4. ed. H.H. Hiatt, J.D. Watson et J.A. Winsten.
- Les Mécanismes de réparation du DNA. Biochimie, 1978, 60.

Produits	Test de mutagénèse *S. typhimurium* *his*$^-$ → His$^+$	Test d'induction de λ *E. coli* K12 (λ)	Conclusions
Aflatoxine B1 Benzo(a)pyrène Nitrosométhyl urée Daunorubicine Sels de platine (II)	+	+	Cancérogènes ou Antitumoraux potentiels
Bromure d'éthidium Azide Ethyl méthanesulfonate	+	−	Cancérogènes potentiels faibles ?
Bléomycine Mitomycine C Actinomycine D	−	+	Antitumoraux potentiels

TABLEAU 1 : Comparaison des tests de mutagénèse et d'induction du prophage λ



Test de mutagénèse ("Spot test") : 10^8 bactéries S. typhimurium (TA100 : his lps uvrB/pKM101) ont été étalées sur des boîtes de milieu synthétique avec 2 ml de gélose molle. Sur les disques de papier filtre mis au centre des boîtes ont été déposés 10 µl d'eau (boîte de gauche) et 10 ul d'éthyl méthanesulfonate (11 mg) (boîte de droite). Les boîtes ont été incubées 2 jours à 37°C.



Test d'induction du prophage λ ("Spot test") : 2000 bactéries lysogènes (GY5027 : envA uvrB (λ)) ont été mélangées avec un homogénat de foie de rat, des cofacteurs, et ont été étalées (volume final de 1 ml) avec 2 ml de gélose molle contenant 10^8 bactéries indicatrices. Sur les disques de papier filtre mis sur la boîte ont été déposés 20 µl de DMSO (Me_2SO) contenant 0, 0,02, 0,2 et 2 µg d'aflatoxine B1. Les boîtes ont été incubées une nuit à 37°C.

MECANISME D'ACTION D'UN CANCEROGENE CHIMIQUE CHEZ E. COLI

Carcinogenesis, Volume 1 October 1980

Cleavage of λ repressor and induction of RecA protein synthesis elicited by aflatoxin B_1 metabolites in *Escherichia coli*

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Abstract

In *Escherichia coli* λ lysogens incubated with activated aflatoxin B_1, the rate of synthesis of RecA protein is markedly increased while λ repressor is cleaved, but not that of the non-inducible mutant λ*cIind*$^-$. Following a 10 min lag period, λ repressor is almost totally cleaved within 40 min of incubation. Cleavage of λ repressor is inhibited by chloramphenicol. Synthesis of RecA protein and cleavage of λ repressor are two characteristic processes induced in *E. coli* by DNA damaging agents such as u.v. light or mitomycin C. Our results favour the idea that in aflatoxin B_1-treated lysogens, DNA lesions activate RecA protein to cleave LexA protein, the repressor of the *recA* gene, and λ repressor. DNA damage, which may represent a relatively small fraction of the total cellular lesions, promotes the derepression of specific genes that control epigenetic as well as genetic processes in bacteria.

Introduction

There is a prevalent concept that cancer is due to heritable changes in the *expression* of the genetic material of a cell (1). The correlation between chemical carcinogenesis in animals and mutagenicity in prokaryotes (2) has provided support for the hypothesis that cell transformation may result from the accumulation of mutations in the DNA of somatic cells (3).

We have shown that potent chemical carcinogens also provoke epigenetic processes in bacteria. After metabolic activation, chemical carcinogens such as polycyclic aromatic hydrocarbons cause the development of a dormant virus (prophage λ) in *Escherichia coli* K12 (λ) (4, 5).

Since DNA lesions caused by u.v. light induce mutagenesis and prophage induction in *E. coli* (reviewed in ref. 6 and 7), we have assumed that powerful chemical carcinogens may act in bacteria as does u.v. light (5). Chemical carcinogens might damage DNA and therefore induce genetic as well as epigenetic processes, the so-called SOS functions (6). Contrary to certain agents that selectively damage DNA or components of the DNA replication machinery (8), most of the chemical carcinogens (or their ultimate reactive metabolites) damage major cellular molecules: DNA, RNA, and proteins (1). Chemical carcinogens might therefore alter transcriptional or translational controls in a cell by binding to informational molecules such as polymerases (9) or products of regulator genes (10) and simultaneously damage DNA.

Recent knowledge of events occurring at the molecular level in λ lysogenic *E. coli* exposed to u.v.-irradiation or to mitomycin C (MMC)* treatment can be summarized as follows: (i) λ repressor, the λ*cI* gene product, which maintains the prophage state, is inactivated by proteolytic cleavage (11); RecA protein "activated" by ATP and single-stranded DNA directs the cleavage of λ repressor *in vitro* (12-14); and (ii) the rate of synthesis of RecA protein increases markedly (15-22); LexA protein, the repressor of the *recA* gene, is also cleaved *in vitro* by the RecA protease (23-25 – J.W.Little, personal communication). Activation of the RecA protease as a consequence of chromosomal damage appears therefore as an early event in the derepression of the *recA* gene and of prophage λ.

If a chemical carcinogen induces lysogenic bacteria by damaging chromosomal DNA, one should observe the induction (i) of the synthesis of RecA protein and (ii) of the cleavage of λ repressor (11,18,26). This is indeed found when aflatoxin B_1 (AFB_1) is used as a model chemical carcinogen.

AFB_1, a product of the mold *Aspergillus flavus* or *A. parasiticus*, is one of the most potent liver toxins and carcinogens for the rat as yet discovered and it may cause cancer in humans (27). AFB_1 is active only after metabolism. Ultimate derivatives, such as AFB_1-8,9-oxide bind covalently to cellular macromolecules (reviewed in ref. 1 and 28). Activation of AFB_1 can be reproduced *in vitro* with a reconstituted mammalian metabolizing system (29-31). Activated AFB_1 is toxic (32) and mutagenic (33) to bacteria, and induces prophage λ in *E. coli* lysogens (34).

We have shown that the λ-inducing concentration of AFB_1 is considerably reduced when λ lysogens carry the *envA* and *uvrB* mutations (4, 5). The *envA* mutation decreases the cell permeability barrier.

*Abbreviations: AFB_1, aflatoxin B_1; MMC, mitomycin C; CAP, chloramphenicol; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Since *envA* mutants form chains of cells, we used *env*$^+$ bacteria in this work in order to avoid artefacts that would make meaningless the comparison of biochemical and microbiological measurements. The *uvrB* mutation is known to prevent excision repair; in *uvrB* mutants, the ratio of damage to DNA over that to other macromolecules is therefore artificially increased. In order not to favour the biological effects of DNA damage, we used *E. coli* strain K12 C600 which is proficient in excision repair.

Our results provide evidence that DNA damage produced by AFB_1 metabolites activates RecA protein to cleave LexA and λ repressors. DNA damage promotes the derepression of specific genes that control epigenetic as well as genetic processes in bacteria.

Materials and Methods

Bacterial and phage strains

Lysogenic derivatives of *E. coli* strain C600 were constructed with phages λ wild type (GY429), *λimm434* (GY479), or *λcIind*$^-$ (GY5010); the *ind*$^-$ mutation located in the *cI* repressor structural gene renders λ repressor insensitive to cleavage (11).

Phage λ was detected as plaques on a lawn of indicator bacteria GY4015, an ampicillin resistant derivative of strain C600 (4).

Strain KM1842 carries the ColE1 hybrid plasmid pLC1842 containing the *recA* gene. This strain is deleted for the chromosomal *recA* gene and carries the *spr-51* mutation in the *lexA* gene, which promotes expression of RecA protein at a high level (35). GY4915 is a derivative of strain KM1842 which has lost the pLC1842 plasmid.

Strain 294/pKB280 contains a plasmid that confers to the host bacteria a tetracycline resistant phenotype and a cellular level of λ repressor 150 times higher than in a λ lysogen (36).

Phage *λprecA* is a plaque forming phage transducing the *recA* region. Phage stocks were prepared by thermal induction of lysogens AB2462(*λcI857 precA*) (15).

Media

YM9 buffer contained : Na_2HPO_4, 41 mM; KH_2PO_4, 22 mM; NH_4Cl, 18.5 mM; NaCl, 85.5 mM; $MgCl_2$, 0.5 mM; $CaCl_2$, 0.1 mM; $MgSO_4$, 1 mM. Enriched YM9 medium was YM9 supplemented with: glucose, 0.2%; thiamine, 1 μg/ml; 14 L-amino acids except the sulfur containing amino acids at 50 μg/ml each. Low sulfur-medium was basically enriched YM9 medium with only 50 μM $MgSO_4$. The division time of bacteria grown in either medium was 45 min at 37°C.

Other media used for the inductest experiments (LB broth, GT-amp medium plates, and soft agar) were as described previously (4, 5).

Chemicals.

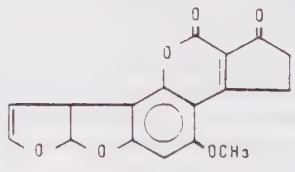
AFB_1, MMC, NADP, and glucose-6-phosphate were from Sigma. Arochlor-1254 was a gift from Monsanto Chem.

In vitro activation of AFB_1

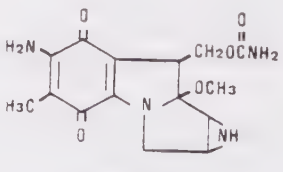
As most chemical carcinogens, AFB_1 and MMC (Figure 1) act on cellular macromolecules only after metabolic activation (27, 37). Bacteria can carry out some of these activating reactions: lysogens are induced by MMC alone but not by AFB_1 alone (34). Therefore, bacteria and AFB_1 were incubated in a reconstituted *metabolizing mixture* consisting of: 13.3% (v/v) of enriched YM9 medium; 80% (v/v) of a NADPH − generating system (0.15 M phosphate buffer at pH 7.5 containing 3 mM NADP, 10 mM glucose-6-phosphate, and 12 mM $MgCl_2$); and 6.7% (v/v) of a rat liver homogenate in 0.15 M KCl, called S-9 fraction. The S-9 fractions were prepared from male Wistar rats pretreated with Aroclor-1254 (38). Aroclor-1254, a polychlorinated biphenyl mixture, is a potent inducer of the various forms of liver microsomal cytochromes P-450 (39), which are implicated in the metabolism of AFB_1 (40). The protein contents of the S-9 preparations were between 25 and 30 mg/ml.

New inductest procedures

The activity as λ-inducer of a chemical carcinogen depends on three major parameters : (a) its concentration in the assay mixture (41), (b) the way it is metabolized, and (c) the genetic constitution of the tester lysogens (4, 5). We have recently observed that



Aflatoxin B1



Mitomycin C

Fig. 1. Chemical structures of AFB_1 and MMC.

the λ-inducing activity of a chemical carcinogen may also depend on (d) the concentration of the treated bacteria, and (e) the aeration of the incubation medium (Moreau *et al.*, manuscript in preparation).

The experiments were therefore performed under the following conditions except where otherwise noted. Bacteria were used at 1–2 x 10^8 per ml, a concentration that provided the required quantities of proteins for RecA and λ repressor titrations. The reaction mixtures were well aerated by vigorous agitation.

A modified procedure of the previously described Inductests (4) was used to measure the λ-inducing activity of activated AFB_1 or MMC at high cell concentration. Assuming that bacteria, whether lysogenic or not, metabolize equally, tester lysogens were diluted not into fresh medium but into exponentially growing 294/pKB280 bacteria used as carrier cells. As a result 10^3 tester lysogens were mixed with 2 x 10^8 carrier cells per ml in the assay mixture (Inductest IVc). Due to their high cellular level of λ repressor the 294/pKB 280 carrier cells are immune to phage λ. One ml portions of the assay mixture were incubated and shaken for 30 min with increasing amounts of the tested chemical, they were then poured onto GT-amp plates with λ-indicator bacteria GY4015. After overnight incubation of the plates, each lysogen which had liberated a progeny of phages formed a plaque on a lawn of indicator bacteria. Plaque counts ranged from about 10 (the background for non-induced lysogens) up to 10^3 when all the treated lysogens were induced.

Inductest IV defines the procedure in which the tester lysogens were diluted into fresh medium without the carrier cells.

Slab gel electrophoresis

Labeled samples were dispersed in final sample buffer (0.125 M Tris.HCl at pH 6.8 containing 2% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercaptoethanol, and 0.0015% bromophenol blue), heated for 2 min at 100°C and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli and Favre (42). After electrophoresis, proteins were fixed in the gel for 1 h with 50% methanol–10% acetic acid; the gels were finally dried and placed in contact with Kodirex X-ray films for 3 to 15 days. The films were then developed using Kodak X-ray developer and rapid fixer. The optical density of the bands of interest was measured on the autoradiograms with a double beam automatic recording microdensitometer (Joyce Loebl and Co.).

Preparation of sera directed against purified RecA protein or λ repressor.

RecA protein was purified as described by Weinstock *et al.* (35), except that bacteria were treated with 5 μg/ml MMC. RecA protein was more than 90% pure as judged by SDS-PAGE (Figure 2). The two contaminants observed had a molecular weight higher than 80 Kd; they accounted for less than 5% of the total protein.

The repressor of prophage λ was purified essentially as described elsewhere (43): in short, bacteria 294/pKB280 were grown up to an $A_{650} = 1$ in low sulfur medium supplemented with 0.2 mM $MgSO_4$ and 20 μg/ml tetracycline. The cells were lysed by sonication, DNA and DNA-bound proteins were precipitated by addition of 0.6% polymin P (Miles), λ repressor was resuspended from the pellet in 0.6 M KCl, precipitated by addition of 0.4 g/ml ammonium sulfate, and purified by CM-Sephadex and hydroxylapatite chromatography. At this point, λ repressor characterized by its specific binding activity to λ operator DNA (data not shown) was found pure at more than 95% as judged by SDS-PAGE and there was no contaminant amounting to more than 1% of the total protein (Figure 2).

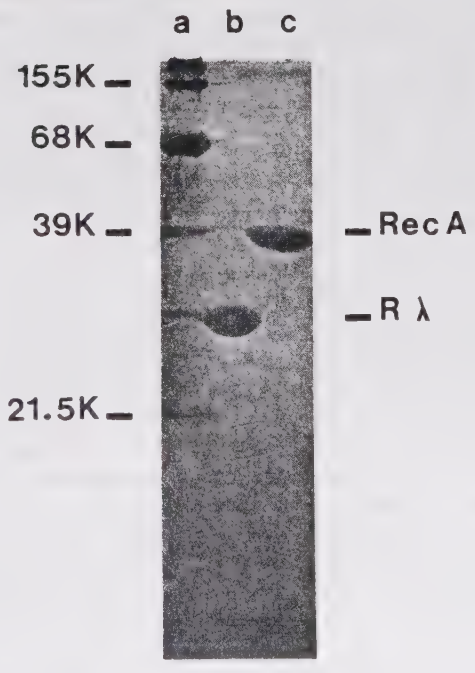


Fig. 2. SDS-PAGE of purified RecA protein and λ repressor. A mixture of proteins (Boehringer) with known molecular weights (lane a), purified λ repressor (20 μg) (lane b), and purified RecA protein (14 μg) (lane c) were analyzed by SDS-PAGE on a 10% acrylamide gel. After electrophoresis, the proteins were stained with 0.005% Coomassie Brillant blue.

Antiserum against RecA protein or λ repressor was prepared as described elsewhere (44).

Measurement of RecA protein synthesis

As a function of the time of incubation of *λcInd*− lysogens with activated AFB_1, or MMC as a control, 200-μl portions were pulse-labeled for 3 min with [^{35}]S-methionine (specific activity 500 Ci/mmol, New England Nuclear). At the end of the labeling period, 0.5 ml of cold YM9 were added and the cells were quickly frozen. Total proteins, membrane proteins, and proteins immunoprecipitated by an anti-RecA serum were then analyzed by SDS-PAGE.

Whole cells and membrane fractions were prepared for electrophoresis essentially as described by Gudas and Pardee (17); for the preparation of the membrane fractions, the cells were lysed by the freeze-thaw lysozyme method (11) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) (19).

The immunological purification of labeled RecA protein was carried out as follows, except where otherwise noted : 200 μl samples of labeled bacteria were mixed with 100 μl of non-radioactive carrier cells GY4915 lacking RecA protein, which had been grown in enriched YM9 medium up to an $A_{650} = 1$ and concentrated 20-fold. Crude extracts were prepared according to Roberts and Roberts (11) except that 1 mM PMSF was added to every buffer (19): the cells were pelleted and resuspended in 25 μl of 0.01 M Tris.HCl at pH 7.9 containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.05 M KCl, and 0.6 mg/ml lysozyme (Worthington), and they were subjected to five cycles of freezing and thawing. After addition of 50 μl of 0.01 M Tris.HCl at pH 7.9 containing 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 M KCl, 5% glycerol, 20 μg/ml deoxyribonuclease I (Worthington), and 10 μg/ml ribonuclease A (Worthington), the extracts were incubated at 20°C for 30 min. Debris was removed by centrifugation. The extracts (70 μl) were mixed with 100 μl of 0.05 M Tris.HCl at pH 7.5 containing 2 M KCl, 2% Triton X-100, and 1 mM PMSF plus 5 μl of anti-RecA serum plus 20 μl of protein A−Sepharose CL-4B (Pharmacia). After 2-3 h incubation at 0°C, the suspensions were poured into small columns, and washed 6 times with 0.2 ml of cold 0.1 M Tris.HCl at pH 6.8 containing 0.5 M LiCl, and 1% 2-mercaptoethanol (45). Immune complexes were then eluted with 40 μl of final sample buffer at 45°C and subjected to SDS-PAGE.

Immunological titration of λ repressor

Lysogens, grown overnight in low sulfur medium supplemented with 0.2 mM $MgSO_4$, were diluted 100-fold into the same fresh medium, grown up to an $A_{650} = 0.4$, and then diluted 4-fold into low sulfur medium containing 0.4 mCi/ml $H_2{}^{35}SO_4$ (specific activity 25 mCi/ml, New England Nuclear). The cultures were incubated for a further 70 min (from an $A_{650} = 0.1$ to an $A_{650} = 0.3$) at which time non-radioactive 10 mM $MgSO_4$ was added for a 5 min chase (11). The labeled bacteria were incubated with different chemicals as described in the legends of the figures. As a function of the time of incubation, 1 ml samples were mixed with 0.5 ml of non-radioactive *λimm434* carrier lysogens and quickly frozen (26). The carrier lysogens had been grown in low sulfur medium supplemented with 0.2 mM $MgSO_4$ up to an $A_{650} = 1$ and concentrated 40-fold. By adding an excess of carrier cells to the labeled lysogens, the background radioactivity was reduced 10-fold without affecting the amount of labeled λ repressor measured on the gels (data not shown). The procedures used for λ repressor immunoprecipitation were essentially as described for RecA protein immunoprecipitation except that (i) PMSF was not added to the buffers, (ii) 1 μl of anti-repressor serum was used, and (iii) incubation was for 12 h at 0°C (11).

Results

Drug activity dependence on cell concentration and on aeration

In order to determine rapidly the optimal concentration of AFB_1 that induces prophage λ at a cell density required for biochemical measurements, that is 10^8 bacteria per ml, exponentially growing lysogens were diluted into carrier cells to obtain 10^3 lysogens among 10^8 carrier cells per ml. This newly devised procedure is different from a previously described assay in which lysogens were incubated with the tested drug at a final cell concentration of 10^3 per ml (4, 5). The two assays are designed to avoid time consuming dilutions in determining the λ-inducing activity of a chemical carcinogen. In performing such different assays with or without addition of carrier cells, we have observed (Moreau *et al.*, manuscript in preparation) that the λ-inducing activity of some chemical carcinogens can vary according to the concentration of the treated cells or the aeration of the assay mixture (see below).

When 10^3 lysogens were mixed with 10^8 carrier cells per ml of metabolizing mixture and incubated with vigorous agitation for 30 min with increasing concentrations of AFB_1, 80% of the treated lysogens were induced at AFB_1 concentrations ranging from 50 to 200 μg/ml (Figure 3). There was no prophage induction when the S-9 fraction was omitted (Figure 3) (34). AFB_1 at a concentration of 100 μg/ml was

subsequently used in conjunction with a metabolizing mixture.

MMC, a potent λ-inducing drug (11), served as a control of AFB_1 activity. MMC was active with or without external activation: the treated lysogens were induced at concentrations ranging from 5 to 10 μg/ml of MMC (Figure 3). MMC was subsequently used without external activation at a final concentration of 5 μg/ml, conditions prevalently used in other laboratories (11, 26).

The optimal λ-inducing concentration of MMC increased up to 50 μg/ml when 10^3 lysogens per ml without carrier cells were put in contact with the drug (Inductest IV) (data not shown). We observed, however, that MMC at a concentration of 5 μg/ml induced all the lysogens whatever their proportion in 10^8 bacteria per ml: either 10^8 lysogens per ml (data not shown) or 10^3 in 10^8 non-lysogens per ml (Figure 3).

We did not observe such a cell density effect upon induction by activated AFB_1 (data not shown). The presence of the S-9 fraction may counteract the effect of cell density.

It should be noted that, if the reaction mixtures containing the tester lysogens in carrier cells and the chemical to be tested were incubated *without* shaking for 30 min before plating, the optimal λ-inducing concentration of activated AFB_1 increased 8-fold, whereas that of MMC decreased 3-fold (data not shown).

To summarize, experimental conditions that tended to decrease the concentration or the diffusion of oxygen in the reaction mixtures reduced about 8-fold the λ-inducing activity of activated AFB_1, but increased up to 30-fold that of MMC.

These results support the hypothesis that the λ-inducing metabolites of AFB_1 are *oxygenated* derivatives (27-31). In contrast, MMC may be activated in bacteria by *reduction* to a free radical intermediate, which either binds covalently to DNA or produces reduced forms of oxygen (O_2^-) (46). However toxic O_2^- is efficiently detoxified by cellular enzymes (47). A low intracellular level of pO_2 may prevent O_2^- generation (48) and increase the formation of MMC-DNA adducts, thus facilitating the λ-inducing process.

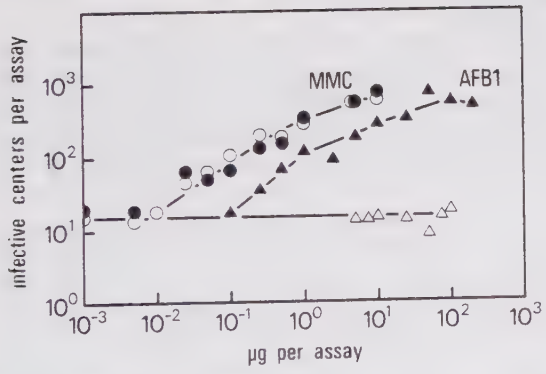


Fig. 3. Dose-effect response curve in Inductest IVc. C600(λ) lysogens grown in enriched YM9 medium up to an $A_{650}=0.3$ (2 x 10^8 cells per ml) were diluted 10^4-fold into the same fresh medium. One ml of this dilution was mixed with 1 ml of 10-fold concentrated 294/pKB280 carrier cells grown up to an $A_{650}=0.5$, 12 ml of an NADPH-generating system, and 1 ml of S-9 fraction: 1 ml portions (containing about 10^3 lysogenic bacteria among 10^8 carrier cells) were then distributed into test tubes containing DMSO (0.05%) with increasing concentrations of AFB_1 (▲), or distilled water with increasing concentrations of MMC (●). The reaction mixtures were shaken for 30 min, mixed with 2 ml of soft agar containing 0.2 ml of GY4015 indicator bacteria, and then poured onto GT-amp plates. When the S-9 fraction was excluded (open symbols), it was replaced by the same volume of LB double strength. Plaques were counted after overnight incubation of the plates.

Activated AFB_1 induces RecA protein synthesis

In order to test whether the synthesis of some bacterial proteins was modified in AFB_1-treated cells, C600 (λ*cIind*$^-$) lysogens, whose prophage is not inducible (34), were pulse-labeled with

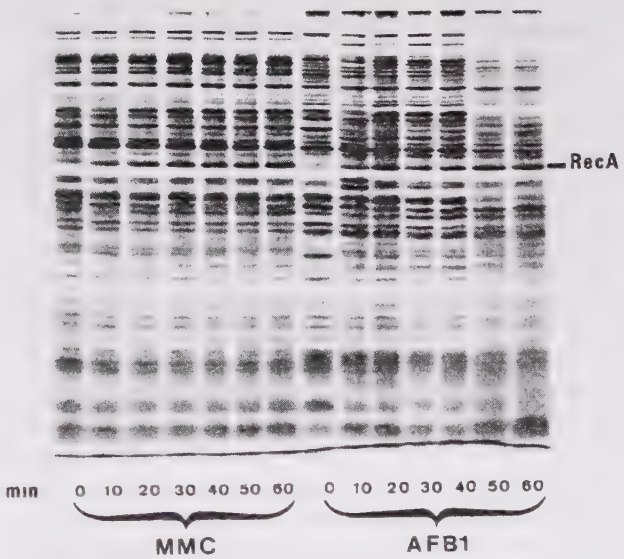


Fig. 4. Induction of RecA protein synthesis: analysis of whole cells. C600 (λ*cIind*$^-$) lysogens grown in enriched YM9 medium up to an $A_{650}=0.3$ were pelleted, resuspended in metabolizing mixture (or enriched YM9 medium), and incubated for 5 min before the addition (zero time) of 100 μg/ml AFB_1 (or 5 μg/ml MMC). As a function of the time of incubation, 200 μl samples were removed, pulsed labeled with 10 μCi/ml [^{35}S]-methionine for 3 min, and analyzed by SDS-PAGE (on a 12% acrylamide gel) and autoradiography. The radioactivity per slot was adjusted to 5 x 10^4 c.p.m. In the first and last slots were extracts of heavily u.v.-irradiated C600(λ*cIind*$^-$) lysogens infected with phage λ*precA* and pulse-labeled with 10 μCi/ml [^{35}S]-methionine; samples contained 7 x 10^3 c.p.m.

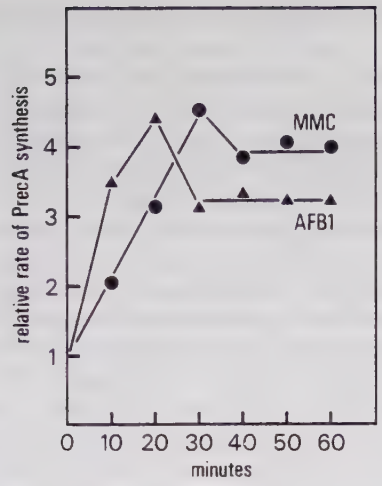


Fig. 5. Time course of induction of RecA protein synthesis. The amount of RecA protein measured on the autoradiogram (Figure 4) by densitometer tracing of the RecA band was taken as a measure of the rate of synthesis of RecA protein at the time of the pulse-labeling. The rate of synthesis of RecA protein normalized to the background level of untreated bacteria was plotted versus the time of incubation with activated AFB_1 (▲) or MMC (●).

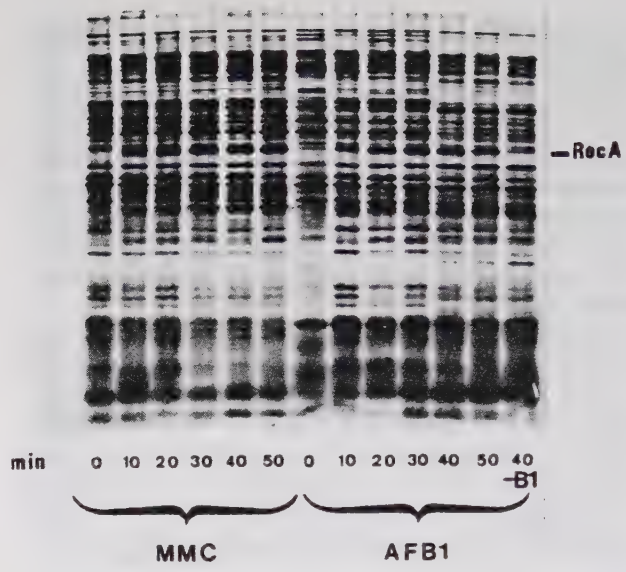


Fig. 6. Induction of RecA protein synthesis: analysis of membrane fractions. Procedure for cell labeling was as in the legend of Figure 4 except that 7.5 ml samples were used. The membrane fractions were prepared for electrophoresis as described in **Materials and Methods.** The radioactivity per slot was 3×10^4 c.p.m.

[^{35}S]-methionine at various times of incubation with activated AFB_1.

AFB_1 treatment provoked a sharp increase in the rate of synthesis of a polypeptide of approximately 39 Kd, which co-migrated with RecA protein made by the transducing phage *λprecA* (Figure 4). The rate of synthesis of this 39 Kd polypeptide reached a peak at 20 min of incubation and then fell. The final level was, however, 3- to 4-fold higher than that observed in untreated bacteria (Figure 5). This 39 Kd polypeptide band did not increase when the S-9 fraction or AFB_1 was omitted from the incubation medium (data not shown). A band of similar size was markedly increased upon incubation with activated AFB_1 when the membrane protein fraction was analyzed (Figure 6).

The polypeptide whose synthesis was induced by activated AFB_1 displays the molecular weight, the time course of induction, and the cellular location of RecA protein induced by MMC (18) (Figures 4-6). That it is indeed RecA protein was proved by specific immunoprecipitation (Figure 7). Note that the RecA protein band was more abundant in KM1842 bacteria overproducing RecA protein (Figure 7); it was not detected in GY4915 bacteria in which the *recA* gene is deleted (data not shown); the band of radioactively labeled RecA protein diminished when an excess of purified unlabeled RecA protein was added as a competitor during the precipitation reaction (Figure 7).

Figure 7 shows that the rate of synthesis of RecA protein was rather low in untreated bacteria although there was a slightly detectable basal level. The rate of synthesis of RecA protein increased about 10-fold or 25-fold upon AFB_1- or MMC-induction as estimated by densitometer tracing.

Note also that three other polypeptides of molecular weight lower than that of RecA protein were also detected on gels (Figure 7). They may be degradative products of RecA protein (19) formed as a consequence of the way the cells were lysed since: (i) they were not detected when whole cell extracts were analyzed (Figure 4), (ii) unlabeled RecA protein competed efficiently with these polypeptides in the immunoprecipitation reaction (Figure 7), (iii) the new polypeptides appeared upon immunoprecipitation of RecA protein produced by phage *λprecA* (data not shown), (iv) the fraction of stable RecA protein did not depend upon the inducing treatment as observed with KM1842 cells containing a high cellular concentration of RecA protein (Figure 7).

Proteolysis of RecA protein seems to occur when it is detached from DNA. The stable RecA protein fraction recovered did not vary with the extent of the incubation period from 2 to 14 h with the antiserum. It was not enhanced by the use of various protease inhibitors (PMSF, N-tosyl-L-phenylalanyl-chloromethane, N-tosyl-L-lysylchloromethane hydrochloride, antipain). The fraction of stable RecA protein increased twice when RecA protein was

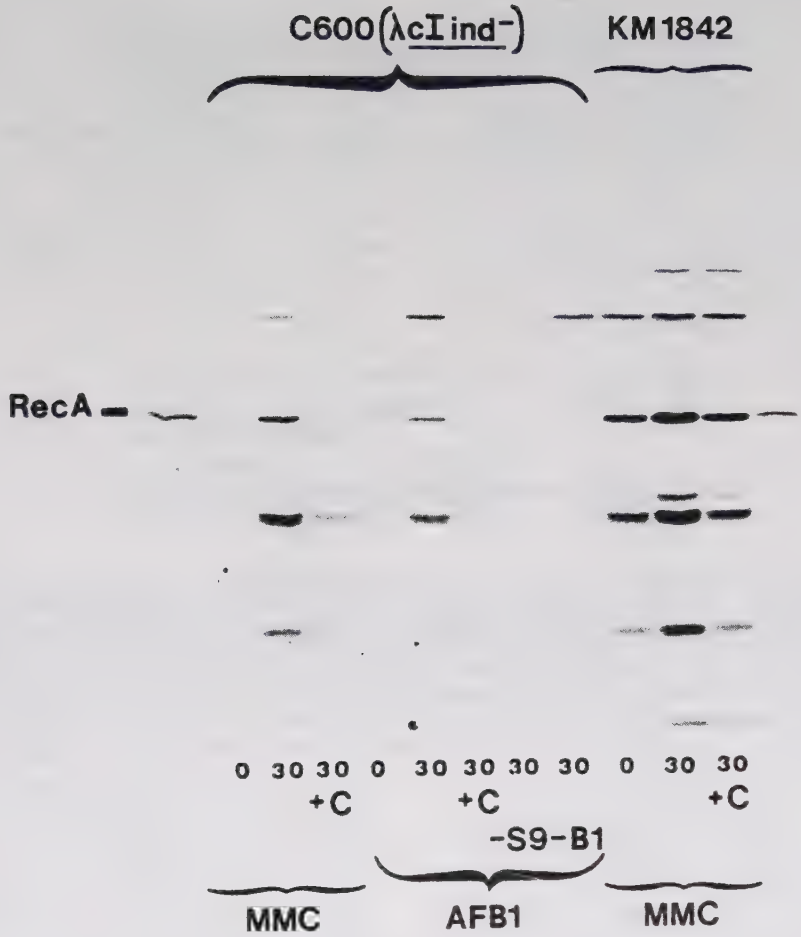


Fig. 7. Immunological measurement of RecA protein synthesis in whole cells. Bacteria incubated with activated AFB_1 or MMC for various times were pulse-labeled as described in the legend of Figure 4, processed for RecA protein precipitation by an anti-RecA serum, and analyzed by SDS-PAGE (on a 10% acrylamide gel). Purified RecA protein (5 μg) was added to the antiserum as a competitor where indicated (+C). Equal volumes were loaded on the gel. Bacteria infected with phage λ*precA* as described in the legend of Figure 4 were placed in the first and last slots.

detached from the bulk of DNA by addition of salt at high concentration plus detergent, the incubation step with nucleases being omitted (data not shown). RecA protein, in contrast to λ repressor (see below), seems subject to a limited proteolysis when not bound to DNA. The biological significance of the limited proteolysis observed is presently investigated.

Activated AFB_1 induces full λ repressor cleavage

When LexA protein, the repressor of the *recA* gene, is modified as a consequence of a *spr*⁻ mutation, the synthesis of RecA protein is increased (20) (Figure 7). This does not result, however, in the cleavage of λ repressor. In the absence of DNA damage, a high concentration of RecA protein is insufficient in itself to induce prophage λ (12, 19). RecA protein must also be activated to act as a protease (11-14).

Cleavage of λ repressor in AFB_1-induced lysogens would therefore demonstrate the conversion of RecA protein into protease.

λ repressor being at a low cellular concentration, its titration requires the use of a specific antiserum (11). λ repressor was finally identified on autoradiograms (R band) by the following criteria: (i) it co-migrated with the only visible labeled polypeptide precipitated from an extract of 294/pKB280 bacteria overproducing λ repressor, (ii) it was not detected if an excess of purified non-labeled λ repressor was added to the serum (data not shown).

It is clear that the cellular concentration of λ repressor decayed exponentially after a 10 min lag period when lysogens were incubated with activated AFB_1 (Figures 8 and 9); more than 80% of λ repressor was destroyed within 40 min of incubation.

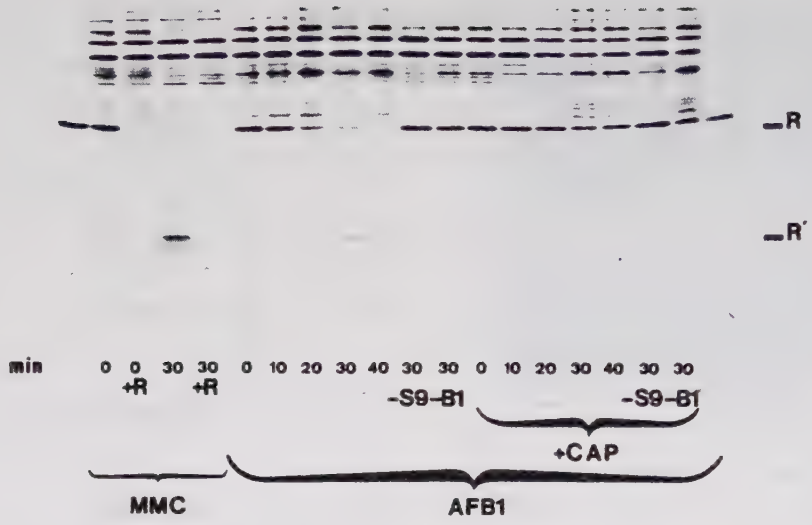


Fig. 8. Cleavage of λ repressor. C600(λ) lysogens at an $A_{650}=0.1$ were incubated with 0.4 mCi/ml $H_2{}^{35}SO_4$ up to an $A_{650}=0.3$ before the addition of 10 mM cold $MgSO_4$, and 100 μg/ml CAP where indicated (+CAP). The cultures were incubated for a further 5 min and pelleted by centrifugation; the bacteria were resuspended at the same concentration in the metabolizing mixture, supplemented or not with 100 μg/ml CAP (+CAP). The zero time samples were taken and the bacteria were further incubated with 100 μg/ml AFB_1. As a function of the time of incubation, 1 ml samples were removed, processed for precipitation of λ repressor by an anti-repressor serum, and analyzed by SDS-PAGE (on a 15% acrylamide gel) and autoradiography. In the reaction mixture, S-9 fraction or AFB_1 was omitted (−S9)(−B1) where specified. Purified λ repressor (4 μg) was added with the serum where indicated (+R). Lysogens were treated with MMC as described in the legend of Figure 10. The radioactivity per slot was adjusted to 5×10^3 c.p.m. Immunoprecipitated proteins from 294/pKB280 bacteria overproducing λ repressor were placed in the first and last slots.

The cellular level of λ repressor was not significantly modified when AFB_1 or the S-9 fraction was omitted from the incubation medium (Figure 8).

Simultaneously to the disappearance of the λ repressor monomer, its cleavage fragments were produced (Figure 8). The cleavage fragments of λ repressor also produced in u.v.- or MMC- induced lysogens (11) (Figures 8 and 10) were evident as a new band called R′: (i) it was approximately one-half the size of the λ repressor monomer (11), (ii) the two bands R and R′ faded out almost totally when purified λ repressor was added as a competitor during the immunoprecipitation reaction (data not shown).

The specificity of the cleavage reaction occurring in AFB_1- induced lysogens was further demonstrated by showing that the modified *cIind*$^-$ repressor, which is not cleaved after u.v. or MMC treatment (11) (Figure 10), was also not cleaved after cell treatment with activated AFB_1 (data not shown).

Cleavage of λ repressor was slightly faster after MMC than after AFB_1 induction (Figures 9 and 10). Note that a 5 to 10 min lag period preceded the onset of λ repressor cleavage. The same time course of decay was observed when λ repressor activity was monitored by a repressor-operator DNA binding assay after induction of lysogens with u.v. light or MMC (49).

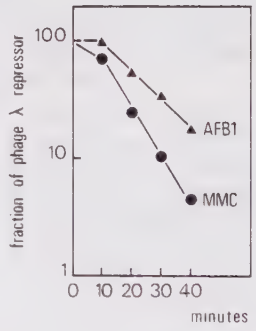


Fig. 9. Time course of λ repressor cleavage. The amount of λ repressor was measured on autoradiograms (Figures 8 and 10) by densitometer tracing of the R band. The plotted values on the ordinate are expressed in percentage of λ repressor in not treated cells. On the abscissa is indicated the time of incubation with activated AFB_1 (▲) or MMC (●).

Cleavage of λ repressor requires de novo protein synthesis

In MMC-induced lysogens, complete inactivation of λ repressor, monitored either by loss of DNA binding activity (49) or by cleavage (26) (Figure 10), requires *de novo* protein synthesis. Protein synthesis is clearly necessary for the activation of RecA protein to act as a protease and may be necessary for increasing slightly its synthesis (50,51).

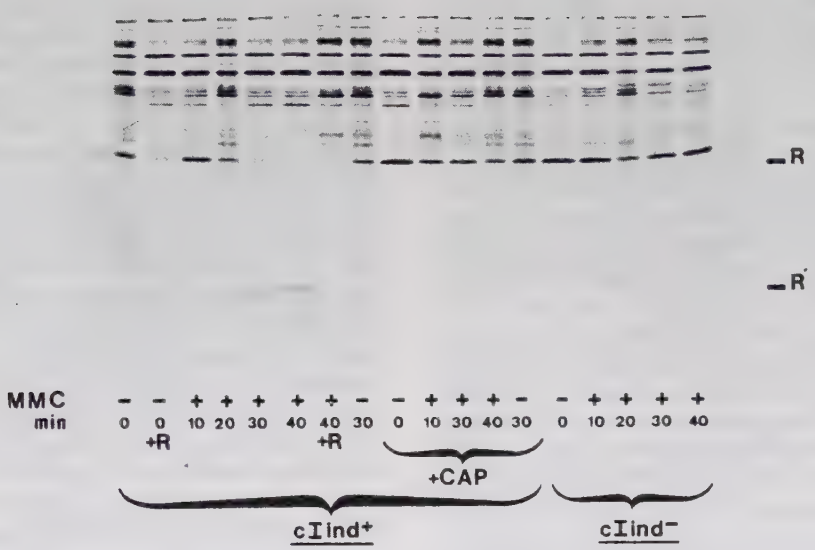


Fig. 10. Cleavage of λ repressor after MMC treatment. C600 (λ) or (λ*cIind*$^-$) lysogens were grown in low sulfur medium containing 0.4 mCi/ml $H_2{}^{35}SO_4$ up to an $A_{650} = 0.3$ before the addition of 10 mM cold $MgSO_4$, and 100 μg/ml CAP where specified (+CAP). The cultures were incubated for a further 5 min, pelleted, and resuspended in enriched YM9 medium containing CAP and MMC, or MMC alone. As a function of the time of incubation, 1 ml samples were removed and processed for λ repressor precipitation by an anti-repressor serum: purified proteins were then analyzed by SDS-PAGE (on a 15% acrylamide gel) and autoradiography. Purified λ repressor (4 μg) was added to the serum as indicated (+R).

Cleavage of λ repressor in AFB_1-induced lysogens requires *de novo* protein synthesis. When bacteria were preincubated with 100 μg/ml chloramphenicol (CAP) for 5 min before the addition of AFB_1, the residual protein synthesis was 5% of the untreated control as measured by a pulse-labeling with [^{35}S]-methionine (data not shown). Under these conditions, cleavage of λ repressor was severely hampered (Figure 8).

Discussion

When lysogens were exposed to an optimal dose of activated AFB_1 (i) the rate of synthesis of the bacterial RecA protein was markedly increased, (ii) the repressor of prophage λ was cleaved, but not that of the non-inducible mutant λ*cIind*$^-$. Cleavage of λ repressor started after a 10 min lag period; at 40 min (an incubation period equivalent to about one generation time for non-induced bacteria) more than 80% of λ repressor was cleaved. That the λ repressor cleavage reaction was the result of an inducible process was clearly shown by the lack of λ repressor cleavage when protein synthesis was blocked by chloramphenicol.

Induction of RecA protein synthesis and cleavage of prophage λ repressor are two characteristic processes triggered in *E. coli* by DNA damaging agents such as u.v. light or MMC (11, 16, 17, 18, 26).

Accumulated lines of *in vivo* and *in vitro* evidence support the hypothesis that λ repressor is cleaved by an activated form of RecA protein. Proteolytic cleavage of λ repressor occurs *in vitro* with purified RecA protein, it also requires ATP and single-stranded polynucleotide molecules (14). Induction of prophage λ takes place in u.v.- or MMC-treated lysogens even though full induction of RecA protein is prevented (50, 51). A high level of RecA protein is insufficient in itself to promote prophage λ induction (12, 19). Such data suggest that activation of the RecA protein to act as a protease and not its increased synthesis is essential for prophage λ induction to occur.

Recently, it has been shown that RecA protein cleaves the *lexA* gene product too (24). LexA protein behaves as the repressor of the *recA* gene (23) as well as the repressor of the *lexA* gene itself (23, 52).

A simple model of the early events following exposure of bacteria to u.v.-irradiation can be formulated. The RecA molecules present in a cell before irradiation are activated by binding to "signal molecules", such as DNA containing single-stranded regions (gaps) (14, 53, 54), generated during repair of damaged DNA. The activated RecA molecules cleave LexA and λ repressors, the synthesis of RecA protein is induced and prophage λ as well. From an evolutionary viewpoint, λ repressor adapted itself to be

sensitive to the bacterial RecA protease so as to escape from a cell doomed to die.

The lag period observed before λ repressor cleavage, but not before induction of *recA* gene transcription (25) and RecA protein synthesis (Figure 5) (17), may mean that cleavage of λ repressor requires a small amplification of RecA protein (51). Alternatively, it may also be that LexA protein being a better substrate for the RecA protease than is λ repressor (24), RecA protein is readily induced while λ repressor is cleaved at a low rate.

It appears that in u.v.-irradiated cells, the amplification of RecA protein and the cleavage of λ repressor are different manifestations of a single event, that is, the transient activation of RecA protein to cleave certain repressors (6). Our results strongly support the hypothesis that the same molecular model applies to AFB_1-induced lysogens.

The hypothesis that AFB_1 metabolites induce prophage λ by a direct alteration of specific sites on DNA, *e.g.*, the *cI* repressor gene or operator binding sites of the repressor, is ruled out by the fact that only the wild-type form of λ repressor was cleaved in AFB_1-induced lysogens.

As most chemical carcinogens, AFB_1 is metabolized into multiple derivatives, which react with many cellular components such as DNA, RNA, and proteins (1), whereas u.v. light and MMC act selectively on DNA. After treatment of *Salmonella typhimurium* with an optimal mutagenic dose of activated AFB_1, Stark *et al.* showed that chromosomal DNA contained only 2% of the total covalently bound AFB_1 molecules (55).

The hypothesis that AFB_1 metabolites specifically interact by covalent attachment, for instance with LexA protein or λ repressor or RecA protein is hardly tenable. In its disfavour are the following arguments. (i) AFB_1 metabolites that bind to DNA are likely different from those that bind to other macromolecules. The major DNA adduct formed in bacteria incubated with activated AFB_1 has been identified as 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1; the same adduct is formed between AFB_1 and rat liver DNA *in vivo* (29, 30). This finding supports the hypothesis that AFB_1-8,9-oxide is the ultimate metabolite which binds covalently to DNA. In contrast, reduced metabolites such as aflatoxicol or closely related derivatives are among the various toxic metabolites damaging macromolecules other than DNA (reviewed in ref. 28). The observation that, under our experimental conditions, the λ-inducing activity of AFB_1 depended on the aeration of the reaction mixture, suggests that the inducing metabolites of AFB_1 were essentially oxygenated derivatives and, therefore, potent DNA damaging agents. (ii) Even though a direct inactivation of lexA protein might mimick the action of a *spr* mutation and therefore trigger the synthesis of RecA protein, this would be insufficient in itself to induce prophage λ since RecA protein must also be activated (12, 19, 54). (iii) The RecA protein can be activated by temperature as a consequence of a *tif* mutation (20, 56). The RecA-Tif protein is a conditional protease (12, 24, 26). Repressor cleavage effected by the RecA-Tif protein is insensitive to chloramphenicol *in vivo* (26). Note that cleavage of λ repressor in AFB_1-induced lysogens was found to be inhibited in lysogens preincubated with chloramphenicol. This result is against the hypothesis that AFB_1 metabolites might directly activate RecA protein as does a *tif* mutation. It also rules out that AFB_1 metabolites might directly inactivate λ repressor by rendering it cleavable by constitutive cellular proteases.

Our present results support the view that, in AFB_1-induced lysogens, even though AFB_1-DNA adducts are scarcely produced as compared to adducts to other macromolecules (28, 55), the activation of RecA protein is a direct consequence of DNA damage.

In *E. coli*, chemical carcinogens provoke DNA damage, a primary *genetic* event, which entails *epigenetic* responses such as the activation of RecA protein and therefore repressor cleavage. The hypothesis has been put forward that proteolytic cleavage of regulatory proteins can account for the various cellular phenomena induced by DNA damage such as cell filamentation, inducible error-prone repair, and prophage induction (6).

In the process of mammalian cell transformation by chemical carcinogens, by analogy with bacteria, DNA damage may be the very event that might entail the expression of normally repressed genes by the activation of specific proteases (57-60).

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MECANISME DES FONCTIONS SOS

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Induction of prophage λ does not require full induction of RecA protein synthesis (*).

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Key words : RecA protein ; λ repressor cleavage ; rifampicin ; mitomycin C.

Résumé.

Les bactéries lysogènes *Escherichia coli* K12 (λ) sont induites par la mitomycine C alors que la vitesse de synthèse de la protéine *RecA* d'*E. coli* est spécifiquement réduite par une faible concentration de rifampicine (4 μg/ml). L'induction du prophage λ a pu être mise en évidence par la lyse des bactéries lysogènes, par la production de phages libres et par la destruction du répresseur du prophage λ. La vitesse avec laquelle se produit le clivage du répresseur et l'efficacité de ce clivage sont pratiquement inchangées bien que la dérépression de la synthèse de la protéine RecA soit fortement inhibée. Toutefois, la production de phages libres est diminuée en présence de rifampicine et la lyse des cellules un peu retardée. On peut donc conclure de ces résultats que chez des bactéries de type sauvage (RecA⁺), l'induction du prophage λ n'exige pas pour se produire la dérépression complète de la synthèse de la protéine RecA.

Summary.

In mitomycin C-treated λ lysogens, even though the rate of synthesis of RecA protein was greatly reduced by a low concentration of rifampicin (4 μg/ml), induction of prophage λ occurred readily as assessed by (i) cell lysis of the lysogens, (ii) production of progeny phage, and (iii) extensive cleavage of λ repressor. The extent and the rate of cleavage of λ repressor were not significantly affected by the low rate of synthesis of RecA protein resulting from rifampicin action. However, the yield of phage progeny was reduced and lysis of the cells was slightly delayed. We conclude that in RecA⁺ bacteria, induction of prophage λ does not require full induction of RecA protein synthesis.

Introduction.

The *RecA* gene in *Escherichia coli* controls genetic recombination [1] and various cellular responses to DNA damage, the so-called « SOS phenomena » [2]. Not only do *recA* mutations affect recombination proficiency but they also prevent inducible error-prone repair (SOS repair), cell filamentation and prophage λ induction from occurring [2]. The product of the *recA* gene [3] was first identified as protein X [4-8], whose rate of synthesis increases sharply when SOS functions are provoked [4, 5, 9].

Induction of prophage λ is one of the most dramatic cellular responses to DNA damage ; its mechanism is presently the most amenable to molecular investigation. In a λ lysogen, prophage λ is maintained in a dormant state by a repressor protein, the product of the prophage *cI* gene [10].

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* *This paper is dedicated to the memory of Jacqueline George.*

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In a monolysogen, when all the λ repressor molecules, present at a concentration of about 140 copies [11], are inactivated [12, 13], and cleaved into two almost equal fragments [14], prophage λ is induced to develop vegetatively. Proteolytic cleavage of λ repressor occurs also *in vitro* with purified RecA protein, it also requires ATP and single-stranded polynucleotide molecules [15-17]. Genetic data [13, 18, 19] support the idea that, *in vivo*, the inactivation of λ repressor results from the expression of a *RecA* gene function.

It has been widely surmised that induction of *RecA* protein is required for prophage induction, as it may be for induction of other cellular responses triggered by DNA damage, such as cell filamentation, inhibition of DNA degradation, or inducible error-prone repair [6, 7, 16, 20, 21]. We report here evidence that full induction of RecA protein is not required for induction of prophage λ in wild-type lysogens.

Materials and Methods.

Strains and growth conditions. Escherichia coli K12 C600 (λ) and C600 (λ*ind*⁻) lysogens were grown in, unless otherwise specified, enriched YM9 medium containing per liter of water : Na_2HPO_4, 7.0 g ; KH_2PO_4, 3.0 g ; NH_4Cl, 1.0 g ; NaCl, 5.0 g ; $MgCl_2$ 0.5 mmol ; $CaCl_2$, 0.1 mmol ; $MgSO_4$, 1 mM ; glucose, 2.0 g ; thiamine, 1 mg ; 14 essential L-amino acids, 50 mg each, the sulfurcontaining amino acids being excluded.

Low sulfur medium was basically enriched YM9 medium with only 50 μM $MgSO_4$.

All cultures were grown or incubated with drugs at 37°C. The turbidity of the cultures was monitored with a Lange photocolorimeter ; a turbidity value of 0.15 corresponded to a cell concentration of 2×10^8 bacteria per ml.

Chemicals. Rifampicin (RIF) (Boehringer) and chloramphenicol (CAP) (Sigma) were dissolved in 0.4 per cent of dimethyl sulfoxide (DMSO). Mitomycin C (MMC) (Sigma) was dissolved in distilled water. L-[^{35}S] methionine (specific activity 500 Ci/mmol) and carrier-free $H_2{}^{35}SO_4$ (specific activity 25 mCi/ml) were obtained from New England Nuclear.

Titration of RecA protein and of λ repressor. RecA protein was titrated in whole cell extracts as described by Gudas & Pardee [5]. RecA protein was identified on autoradiograms by comparing its mobility to that of RecA protein made by the transducing phage λ*precA*⁺ [3] ; following infection of heavily UV-irradiated C600 (λ*ind*⁻) lysogens by λ*precA*⁺, RecA protein expressed by the transducing phage was the only protein labeled during incubation with [^{35}S] methionine.

Cleavage of λ repressor was measured by immunoprecipitation essentially as described by Roberts & Roberts [14] except that the soluble complex λ repressor - antibody (anti-λ repressor serum was always in excess) was not precipitated by addition of a second antiserum but chromatographed on columns of protein A-Sepharose CL-4B (Pharmacia) [22].

Samples were dispersed in « final sample buffer » [23], heated for 2 min at 100°C and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [23] in order to separate labeled proteins. The radioactivity in ^{35}S per slot was adjusted to be the same in each experiment. After electrophoresis, gels were dried and placed in contact with Kodirex X-ray films.

On the autoradiograms, the optical density of the band of interest was measured with a double beam automatic recording microdensitometer (Joyce-Loebl and Co.). Under our experimental conditions a linear relationship was observed between the number of ^{35}S counts in RecA protein (expressed by the transducing phage λ*precA*⁺) and the optical density of corresponding band on autoradiograms. Thus, when the cellular proteins were pulse-labeled with [^{35}S] methionine, the optical density of the RecA band on autoradiograms was a measure of the rate of synthesis of RecA protein at the time of the pulse. As for the optical density of λ repressor band, it corresponded to the amount of λ repressor remaining in the lysogens at a given time of incubation.

Results.

1. *Rifampicin decreases RecA protein synthesis induced by mitomycin C.*

Mitomycin (MMC), a DNA alkylating and cross-linking agent [24], inhibits DNA replication ; it is a potent inducer of prophage λ development as well as of RecA protein synthesis. MMC treatment of *E. coli* is known to cause inducing effects similar to UV-irradiation [9, 12, 14, 25]. Rifampicin (RIF), at a high concentration (80 μg/ml), inhibits RNA polymerase rather specifically. Most notably, RIF, at a low concentration (4 μg/ml), has been reported to prevent selectively the synthesis of RecA protein induced by nalidixic acid treatment [20, 21].

First, we checked to see whether or not RIF at a concentration of 4 μg/ml prevented RecA protein synthesis from being fully induced by MMC treatment. C600 (λ*ind*⁻) lysogens were therefore incubated with RIF first and, 5 min later, with MMC or, as a control, with MMC alone. At various times of incubation, the cellular proteins were pulse-labeled for 3 min with [^{35}S] methionine. The cellular proteins were separated by SDS-PAGE (fig. 1), and autoradiograms were subjected to scanning densitometry to quantitate the rate of synthesis of RecA protein (fig. 2). It is clear that induction of RecA protein by MMC treatment was greatly reduced by the presence of RIF in the

incubation medium (fig. 1). The relative rate of synthesis of RecA protein increased roughly linearly for 30 min from 1 to 4.5 without RIF, and from 1 to 1.5 with RIF (fig. 2). At 30 min of incubation, the rate of synthesis of RecA protein induced by MMC was therefore reduced by RIF by about 77 per cent. In contrast, the overall rate of synthesis of cellular proteins was reduced only by 10 per cent at the same concentration of RIF (data not shown).

by RIF independently of the presence of MMC (fig. 1) but was not affected by CAP (data not shown).

2. *Induced λ lysogens lyse in the presence of RIF.*

Satta *et al.* [21] have reported that RIF at a concentration of 4 μg/ml reduced bacteriophage P1 « induction » in *E. coli* B/r by lowering the induced synthesis of RecA protein. In contrast,

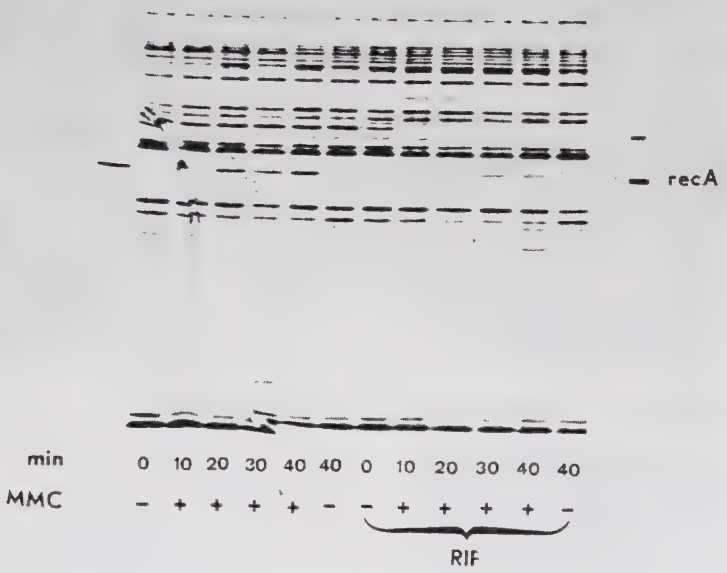

Fig. 1. — *Induction of RecA protein synthesis.*

C600 (λ*ind*⁻) lysogens grown in enriched YM9 medium up to 0.15 Lange units were incubated, or not, with 4 μg/ml RIF for 5 min before addition of 5 μg/ml MMC. As a function of the time of incubation after the addition of MMC, 200-μl samples were withdrawn and incubated further with 10 μCi/ml [^{35}S] methionine for 3 min and quickly frozen after the addition of cold YM9. Labeled cellular proteins were then analysed by SDS-PAGE (on a 12 per cent acrylamide gel) and autoradiography. The radioactivity in ^{35}S per slot was adjusted to 10^5 cpm. Below each lane on the autoradiogram are indicated the times of incubation at which 3-min pulses were started. In the first slot at the left was an extract of heavily UV-irradiated C600 (λ*ind*⁻) lysogens infected with phage λ*precA*⁺ and incubated with 10 μCi/ml [^{35}S] methionine for 30 min ; the sample contained 7×10^3 cpm. The molecular weight of the RecA protein was about 40,000.

When chloramphenicol (CAP) was used at 1.4 μg/ml, a concentration that mimicked RIF effects on cell growth and protein syntheses (data not shown), it did not affect the induction of RecA protein synthesis by MMC (fig. 2).

The synthesis of another protein, not yet identified, of approximately 50 Kd, was also inhibited

we observed full cell lysis of λ lysogens incubated with RIF and MMC, CAP and MMC, or with MMC alone (fig. 3). Although in the first culture (RIF + MMC) cell lysis was slightly delayed and the final phage titer was 2.5-fold lower than in the control culture, there is no doubt that prophage λ was fully induced in all the treated lysogens.

To account for the effect of RIF on phage λ production, we must consider two alternative mechanisms. Either the inactivation of λ repressor was delayed and, consequently, the onset of phage development, or the inactivation of λ repressor was complete but not the repair of the MMC-damaged prophage whose replication, and therefore maturation into progeny phage might have been hampered.

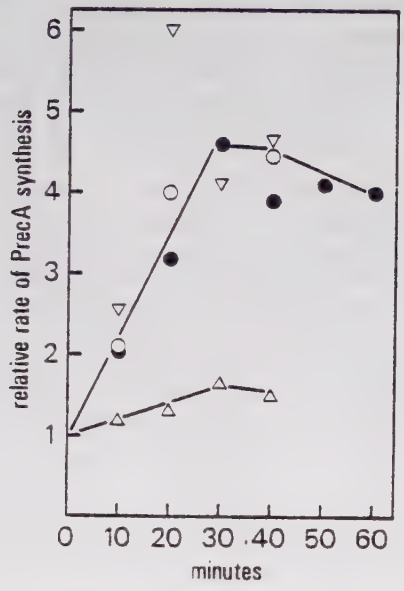

FIG. 2. — *Time course of induction of RecA protein synthesis.*

C600 (λ*ind*⁻) lysogens were pulse-labeled with [^{35}S] methionine (as described in fig. 1) as a function of the time of incubation with RIF and MMC (△), CAP and MMC (▽), DMSO and MMC (○), or MMC alone (●). The relative rate of synthesis of RecA protein was calculated as the ratio of the optical density (on autoradiograms) of the band at a given time of incubation with MMC over that observed before MMC addition. The basal rate of RecA protein synthesis was not significantly different whether the bacteria were preincubated or not with RIF or CAP.

3. *RIF does not affect the rate of cleavage of λ repressor.*

To differentiate between the two mechanisms enunciated above, we measured the time-course of inactivation by cleavage of λ repressor [14].

C600 (λ) lysogens were first incubated for 70 min (time for 1.5 cellular divisions) with $H_2{}^{35}SO_4$; after a 5-min chase by cold $MgSO_4$, in the presence or the absence of RIF or CAP, the lysogens were further incubated for different times with MMC. Samples were then lysed and labeled λ repressor was titrated in the crude cell extracts (figs 4 and 5).

Figure 5 shows that the decay of λ repressor was delayed by approximately 3 min when the lysogens were incubated with RIF and MMC, as compared to lysogens incubated with MMC alone. When the lysogens were incubated with CAP and MMC, the decay of λ repressor was a little more rapid than in the control culture. In any case, a 5 to 10 min lag period preceded the decay of λ repressor and more than 90 per cent of λ repressor was destroyed by 40 min of incubation.

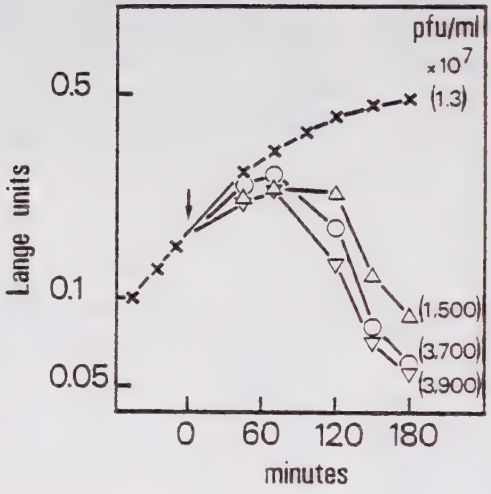

FIG. 3. — *Cell lysis produced by phage λ development.*

C600 (λ) lysogens were grown in enriched YM9 medium up to 0.15 Lange units. The culture was split into 4 samples : (a) was treated with 4 μg/ml RIF, (b) with 1.4 μg/ml CAP, (c) and (d) were left untreated. The four samples were incubated for 5 min and then 5 μg/ml MMC were added (arrow) to (a), (b) and (c), but not to (d). At various times after MMC addition (zero time), the turbidity of the cultures was measured (values higher than 0.3 Lange units are underestimated). After incubation for 180 min, the titer of phage released is indicated in parentheses on the graph. a : RIF + MMC (△) ; b : CAP + MMC(▽) ; c : MMC (○) ; d : untreated (X).

Upon incubation of lysogens with MMC, in the presence or the absence of RIF (fig. 4) or CAP (data not shown), the λ repressor monomer (R) was transformed into characteristic cleavage fragments (R'), which are approximately one-half the size of λ repressor [14, 26]. The amount of R' fragments increased from 10 to 30 min incubation time and declined rapidly thereafter. Note that

the amount of R' fragments seen on the autoradiograms (fig. 4) was slightly increased by the presence of RIF. After 10 min of incubation of the lysogens with RIF and MMC, R' fragments were

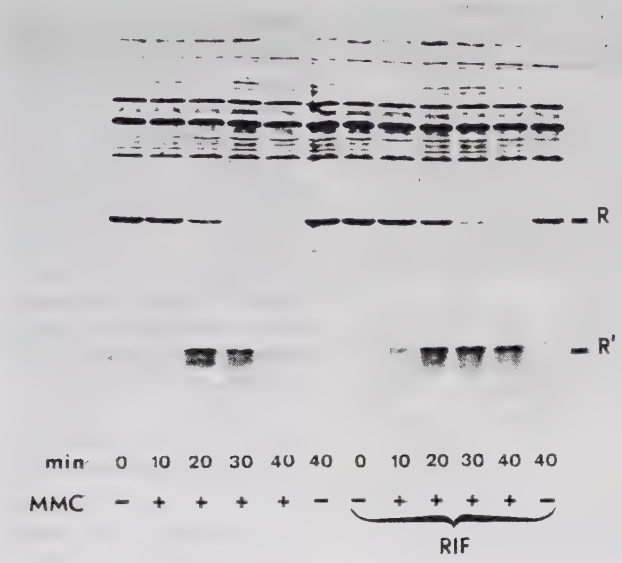

FIG. 4. — *Cleavage of λ repressor.*

C600 (λ) lysogens were grown in low sulfur medium containing 0.4 mCi/ml $H_2{}^{35}SO_4$ from 0.05 to 0.15 Lange units. The culture was then split into two samples : one half was treated with RIF and 10 mM cold $MgSO_4$, to the other half cold $MgSO_4$ was added. The two cultures were incubated for a further 5 min, centrifuged and then resuspended in warm enriched YM9 medium containing RIF and MMC, or MMC alone, as indicated on the autoradiogram. As a function of the time of incubation with MMC, 1-ml samples were removed and processed for λ repressor precipitation by an anti-repressor serum. Purified labeled proteins were analysed by SDS-PAGE (on a 15 per cent acrylamide gel) and autoradiography. The radioactivity in ^{35}S per slot was adjusted to 6×10^3 cpm. Below each lane on the autoradiogram are indicated the times at which the samples were removed and quickly frozen. The molecular weight of the λ repressor monomer (R) and of its fragment (R') were about 27,000 and 14,000 respectively.

more abundant than without RIF, and their disappearance was also delayed ; no similar affect was observed in the presence of CAP (data not shown).

Discussion.

Upon treatment of *E. coli* with DNA damaging agents, the synthesis of RecA protein is induced [4, 5, 9, 27] ; the concentration of RecA protein can reach 3 per cent of the total of the cellular proteins after 50 min of incubation [5]. Such treatments induce also the so-called SOS functions, such as cell filamentation, inhibititon of DNA degradation and prophage induction, the expression of which appears to be coordinately controlled [2]. Does this mean that full induction of RecA protein is required to promote all SOS functions as has been proposed by many authors [6, 7, 16, 20, 21] ?

We show in this publication that this is not so for induction of prophage λ. Indeed, in MMC-treated lysogens, even though the rate of synthesis of RecA protein was quite specifically reduced by a low concentration of RIF, induction of prophage λ occurred readily as assessed by : (i) cell

lysis of the lysogens, (ii) production of progeny phage, and (iii) extensive cleavage of λ repressor.

In particular, the rate and the extent of cleavage of λ repressor were not significantly affected by the decreased rate of synthesis of RecA protein

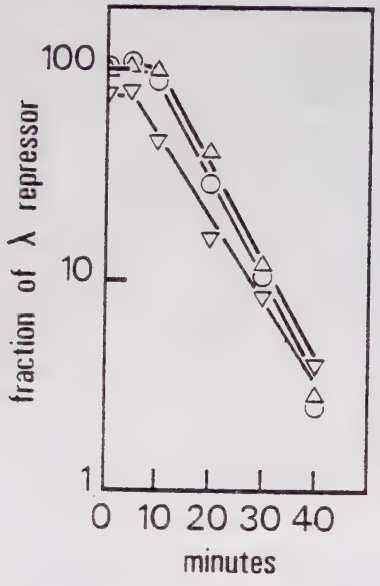

FIG. 5. — *Time course of cleavage of λ repressor.*

C600 (λ) bacteria were incubated as described in figure 4 with RIF and MMC (△), CAP and MMC (▽), or with MMC alone (○). The amount of λ repressor was measured on autoradiograms by densitometry of the repressor (R) band. The plotted values on the ordinate are expressed in percentage of λ repressor in the control culture (RIF and CAP free) just before MMC addition ; they represent the mean of two experiments. On the abscissa is indicated the time of incubation with MMC.

resulting from RIF action. The lag period before the decay of λ repressor was, however, slightly extended in the presence of RIF (fig. 5) and the disappearance of λ repressor fragments (R') was slow (fig. 4) ; in contrast, the time course of production of R' repressor fragments was rather accelerated by RIF (fig. 4). The apparent discrepancy between the two results might reflect the complex cellular effects of RIF at low concentration. It has been shown that RIF at low concentration can stimulate the transcription of some operons and reduce that of others selectively [28, 29]. In our experiments, we might have observed the net result of various effects of RIF. RIF clearly reduced the induced synthesis of RecA protein but might have also delayed the degradation of λ repressor fragments, which is normally occurring rapidly in growing cells [30]. Alternatively, RIF at low concentration might have allowed a higher synthesis of λ repressor during the 5-min chase period before the addition of MMC, whereas CAP at low concentration might have inhibited the synthesis of λ repressor, and therefore accelerated the cleavage of λ repressor (fig. 5) and thus induction of prophage λ (fig. 3).

Notwithstanding, it is clear that cleavage of λ repressor, which is thought to be the terminal cellular event leading to prophage λ induction, did not require full induction of RecA protein.

We do not claim that the basal level of RecA protein is sufficient to cleave λ repressor since RIF did not completely abolish the induction of RecA protein synthesis. Moreover, our data do not exclude the possibility that a background of cellular proteins other than RecA (of similar molecular weight) might have led to underestimate the increase in the rate of synthesis of RecA protein. This is all the more possible as the titration of RecA protein was not performed with a technique as specific as that used for λ repressor titration. Since RecA molecules are stable for at least 1 h [4, 33], the accumulation of RecA protein induced by MMC might have been underestimated in the presence of RIF.

Furthermore, it is known that (i) *de novo* protein synthesis is required for cleavage of λ repressor in MMC-induced lysogens [31], (ii) lexA mutants, in which RecA protein synthesis is poorly induced after DNA damage [32], do not express SOS functions [2], and (iii) the beginning of λ repressor breakdown is preceded by a lag period [12, see also fig. 5]. These facts and our results taken together may simply mean that a small accumulation of RecA protein is necessary to obtain full cleavage of λ repressor.

It may also be that the cellular events preceding the decay of λ repressor are related to the occurrence of DNA repair processes, which may lead to the activation of RecA protein to cleave λ repressor [17].

That RecA protein must be activated for the cleavage reaction to occur is supported by various genetic and biochemical data :

(i) Although RecA protein is produced at a high cellular concentration as a result of multiple *lexA* mutations (*lexA3 spr-51* or *lexA3 tsl-1*) or of amplification due to a plasmid, induction of prophage λ does not occur constitutively, that is, in

the absence of DNA damage [6, 15, 34, 35]. Moreover, in some bacterial mutants, such as *infA*, DNA damage induces RecA protein synthesis, whereas there is no prophage λ induction [9, 36, L. J., Gudas, pers. comm.].

(ii) *De novo* RNA and protein syntheses are required in order for λ repressor to be inactivated in a *tif-1* mutant at 42°C whereas the amplification of RecA protein modified by the *tif* mutation [6-8] is not needed [37]. Thus, in the case of the *tif* mutant, the activation only of the (RecA) Tif protein seems sufficient for prophage λ induction. However, note that : (1) the *tif* mutant synthesizes RecA protein at a faster rate than the wild type strain even at 30°C [32], (2) purified Tif protein is considerably more active than the RecA$^+$ protein as regards cleavage of λ repressor [16]. What applies to the *tif-1* mutand does not apply necessarily to wild type bacteria considered in our experiments.

(iii) Cleavage of λ repressor requires, apart from the purified (RecA) Tif protein, single-stranded DNA and also ATP [17].

If activation of RecA protein is required for cleavage of phage λ repressor, our results indicate that only a small (if any) increase over the background level of RecA protein is necessary to promote total λ repressor cleavage. This may be interpreted as indicating that the molecular signals activating RecA protein, whatever their nature [17], are produced in limited amounts after a DNA damaging treatment. This hypothesis can account in a simple way for the apparent paradox that thousands of RecA molecules must take 40 min (about a generation time) to cleave a hundred or so molecules of λ repressor in an induced λ lysogen. A fraction only of induced RecA molecules may act as a protease [13, 34], in such a way that a reduction in the total level of RecA protein will not affect prophage λ induction, even though other cellular processes, such as DNA repair, may then be inhibited.

Alternatively, in an induced lysogen, there may be an abundance of active RecA protein but a limited amount of λ repressor susceptible to cleavage at a given time. Experiments performed in our laboratory have shown that upon UV-irradiation of λ lysogens, the rate of inactivation of λ repressor did not change in spite of a 5-fold increase due to a plasmid in the cellular level of λ repressor ; the inactivation system, however, did not appear to be saturated [13]. One interpretation of this result is that there are two forms of λ repressor, cleavable and non cleavable, and that there is a dynamic equilibrium between these two forms [13, J. W. Roberts, pers. comm.]. The speed of generation of cleavable (*e.g.*, monomeric) forms drawn from a pool of non cleavable (*e.g.*, oligomeric [26]) forms of λ repressor may be the limiting factor of the cleavage reaction and may render it independent of the cellular level of RecA protein within a relatively broad range.

Finally, we cannot rule out the possibility that RecA protein might not be itself the repressor cleaving enzyme but might be the requirement for another unknown inducible protease to cleave λ repressor. The protease might be present in trace amounts associated with purified RecA protein [17].

Whatever the exact role or function of RecA protein in prophage λ induction is, when the cleavage of λ repressor starts, there is no apparent correlation between the rate of cleavage of λ repressor and the rate of synthesis of RecA protein. Recently, a lack of correlation has also been observed between the synthesis of RecA protein and another SOS function, namely the inhibition of cell division [33].

Note added in proof : While this paper was in press, a paper by Baluch *et al.* (1980) was published whose title is : Induction of prophage λ without amplification of *RecA* protein, *Mol. Gen. Genet.*, 178, 317-323.

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UV-reactivation and induction of prophage λ in Escherichia coli K12 overproducing RecA protein

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SUMMARY

E. coli K12 bacteria overproducing RecA protein and/or carrying a *lexA3* mutation were tested for their ability (i) to survive to UV irradiation (ii) to promote UV-reactivation of UV-damaged phage λ (iii) to induce prophage λ. Bacterial survival was related to the cellular level of RecA protein except in a *lexA3* RecAo (RecA protein overproducer) double mutant in which the *lexA3* mutation slightly sensitized bacterial survival. In this double mutant, UV-reactivation of UV-damaged phage λ was not observed whereas prophage λ was induced to about 40 %. Our results indicate that (i) inactivation of LexA repressor by RecA protease is required not only for the induction of RecA protein synthesis but also for the occurrence of induced DNA repair processes needed for efficient cell as well as phage survival (ii) there is no proportionality between the amount of RecA protein in the cell and the formation of RecA protease.

INTRODUCTION

In Escherichia coli, damage to DNA induces a group of cellular functions regulated by recA and lexA genes called "SOS functions" (Witkin 1976). Among SOS functions, two of them have been extensively studied, inducible error-prone repair, exemplified by UV-reactivation of phage λ, and induction of prophage λ (see reviews by Witkin 1976, and by Devoret 1981).

After a treatment damaging cellular DNA, transcription of the recA gene is stimulated (McPartland et al. 1980) and the cellular level of RecA protein increases highly (Gudas and Pardee 1976). Recently, it has been demonstrated that LexA protein binds to the operator of the recA gene and inhibits recA gene transcription (Little and Harper 1979 ; Brent and Ptashne 1980 ; Little et al. 1981 ; Brent and Ptashne 1981), thus LexA protein represses the recA gene. Moreover, LexA protein is cleaved by RecA protein (Little et al. 1980 ; Little et al. 1981). Until now, RecA protein is the only protein known to cleave the repressor of its own gene.

Induction of SOS functions is prevented by recA$^-$ or lexA$^-$ mutations which also block the induction of RecA protein synthesis (Witkin 1976). The phenotype of lexA$^-$ mutants can be accounted for by the inability of RecA protease to cleave LexA protein (Little et al. 1980).

It has been widely admitted that induction of SOS functions is a consequence of induction of RecA protein

synthesis (McEntee 1977 ; Gudas and Mount 1977 ; Roberts et al. 1978 ; Satta et al. 1979). However, there is a lack of correlation between the synthesis of RecA protein and the induction of prophage λ and filament formation (Baluch et al. 1980 ; Moreau et al. 1980a; Darby and Holland 1979 ; Huisman et al. 1980). These findings led us to question whether the lack of UV-reactivation in lexA⁻ mutants (Defais et al. 1971) was due simply to the absence of induction of RecA protein synthesis (Gudas 1976) or to some other functions controlled by LexA repressor. To test these possibilities, we used strains whose cellular level of RecA protein was constitutively elevated, similar in that respect to the strain characterized by Volkert et al. (1981) ; we also checked the role of the lexA3 mutation.

MATERIALS AND METHODS

Bacterial and phage strains

Escherichia coli K12 derivatives are listed in table 1. Bacteria overproducing RecA protein (see Fig. 3) were of two kinds : (1) Bacteria harboring a ColE1 plasmid carrying the *srl*$^+$ *recA*$^+$ markers (Clarke and Carbon 1976) ; (2) Bacteria of phenotype RecAo, that is overproducing *RecA* protein, may be due to a cis-acting regulatory mutation near or in the *recA* gene (Ginsburg and Mount, to be published).

The molecular evidence for the RecAo phenotype of strain DM2210 appears to be due to a constitutive mutation in the operator region of *recA* gene since : (i) the mutation did not detectably alter the molecular weight of RecA protein as measured on SDS-polyacrylamide gel (data not shown), (ii) the derepression of the *recA* gene was almost maximal in the absence of DNA damage as shown in Figs 3 and 4, and (iii) the proteolytic activity of RecA protein seemed not different from that of the wild-type strain (Fig. 7).

Phage strain λGY15 (Quillardet and Devoret 1981) was used in UV-reactivation experiments. Lysogens carried prophage λ. Phage λ*cI26* and λ*146* (Bailone and Devoret 1978) served to test bacterial immunity to phage λ.

Media, chemicals and cultures

Media were as described in Quillardet and Devoret (1981), and Devoret et al. (1981).

Bacteria were grown in LB medium to which 0.25 % maltose was added when bacteria were to be infected with phage λ. Dilutions were done in 0.01 M $MgSO_4$.

Mitomycin C (MMC) was from Sigma.

Lysogenization of bacteria .

Overnight cultures of bacteria were centrifuged, resuspended at $5x10^8$ cells per ml in 0.01 M $MgSO_4$, and infected at a multiplicity of 1 phage per 400 bacteria. After an 60 min adsorption period at 37°C, the infected bacteria were diluted twice in LB containing 0.01 M $MgSO_4$, and incubated with agitation for about 6 hours. After incubation, the mixtures were diluted in 0.01 M $MgSO_4$. Samples of appropriate dilutions were infected with phage λcI26 and plated on EMBO medium. Non-immune bacteria were lysed by phage λcI26. Prospective lysogens were purified a few times as immune to λcI26 and sensitive to virulent phage λ146.

Test of immunity to colicin E1.

Bacteria carrying a ColE1 plasmid are immune to colicin E1. Immunity to colicin E1 was tested before each experiment so as to be certain that the bacteria had not lost the ColE1 plasmid. Colicin E1 preparation was a chloroformed supernatant of colicinogenic culture treated with MMC ; 0.1 ml of colicin was mixed with 3 ml of soft agar and poured onto LB plates. Master plates with gridded colonies were then replica-plated. Only did plasmid carrying colonies grow after overnight incubation.

Cellular level of RecA protein measured by cell resistance to MMC.

Resistance of bacteria to MMC constituted a rapid test to indicate the cellular level of RecA protein. MMC-plates were prepared as follows. Increasing volumes : 0, 5, 15, 30, 40, 50 or 60 µl of a 1 µg/µl MMC solution were added to 3 ml of soft agar at 45°C and poured onto EMBO plates. Master plates with gridded colonies were then replica-plated and incubated overnight at 37°C.

EMBO medium was used because it permitted to class the tested colonies into 3 categories according to the size and color of replica-plated patches : (i) no growth ; (ii) little growth (patches were dark-purple) ; (iii) normal growth (patches were pinkish-white). Growth ability depended on cell's resistance to MMC and corresponded to the cellular level of RecA protein (compare Fig. 1 and 3).

UV-irradiation .

Bacteria or phage were irradiated with a 15 W germicidal lamp. Doses were measured with a Latarjet dosimeter.

Bacteria and phages were UV-irradiated at doses giving 1 % survival. Bacteria were given the following doses : AB1157, 80 J/m^2 ; DM1623, 6 J/m^2 ; DM2210, 80 J/m^2 ; DM2211, 80 J/m^2 ; JA200/pLC30-20, 80 J/m^2, except when otherwise noted on the figures.

The dose given to phage λGY15 was : 150 J/m^2, except when otherwise indicated on the figures.

Phage assays.

In UV-reactivation experiments, assays for virulent mutants (λvir) and for parental phage λGY15 have been described elsewhere (Quillardet and Devoret 1981).

The number of infective centers produced by induction of prophage λ was determined using the ampicillin method described by Moreau et al. (1976).

Immunological measurement of the cellular level of RecA protein by rocket-immunoelectrophoresis.

The bacterial cultures were grown at 37°C in enriched YM9 medium (Moreau et al. 1980b) up to an A_{650} = 0.3. For each culture, a sample was taken before UV-irradiation (zero dose) and processed for immunoelectrophoresis as described below. The rest of the culture was centrifuged, the pellet resuspended in cold 0.01 M $MgSO_4$, exposed to UV-light, recentrifuged, resuspended in enriched YM9 medium and further incubated with vigorous agitation. Incubation was stopped by adding cold YM9 containing 10 mM NaN_3 . The crude extracts were prepared as described by Roberts and Roberts (1975). Then 10 µl aliquots of the crude extracts were put into the wells of a horizontal 1 % agarose gel containing 5 % anti-RecA serum (Laurell 1966). Electrophoresis was done at 80V for 18 h. The immunoprecipitates were stained with Amido-Black. Under our experimental conditions, we observed a proportionality between the area of the rocket and the quantity of RecA protein (Moreau et al. 1981).

RESULTS

1. Bacterial survival is dependent upon the cellular level of RecA protein.

It is generally admitted that large amounts of RecA protein are required for DNA repair and cell survival (Gudas and Pardee 1976 ; Satta and Pardee 1978 ; Satta et al. 1979). To assess this hypothesis quantitatively, we measured cell survival to UV-light and the induced cellular level of RecA protein in various E. coli K12 strains.

We used strains carrying mutations or a plasmid that affected the basal and induced levels of RecA protein. As to their resistance to UV-irradiation, the strains used have been classified as follows : JA200/pLC30-20 : *lexA*$^+$/ ColE1 *recA*$^+$>DM2211 : *lexA*$^+$ RecAo and AB1157 : *lexA*$^+$ *recA*$^+$> DM2210 : *lexA3* RecAo > DM1623 : *lexA3* *recA*$^+$(Fig. 2).

The amount of RecA protein was measured by the immunoelectrophoresis technique of Laurell (1966) ("rocket immunoelectrophoresis") (Figs 3 and 4). After 90 min of post-irradiation incubation, the differential increase in the cellular level of RecA protein was respectively 1.2-, 1.5-, 3- and 9-fold for *lexA3* RecAo$^{\pm}$, *lexA*$^+$ RecAo, *lexA*$^+$/ColE1 *recA*$^+$, and *lexA*$^+$ *recA*$^+$ bacteria (Fig. 4). This suggests first that production of RecA protein was almost maximal in the absence of DNA damage in *lexA*$^+$ RecAo bacteria as in *spr* bacteria (Fig. 3). The level of RecA protein present in RecAo bacteria was nearly reached by the parental bacteria after 90 min of post-irradiation incubation by induction of the *recA* gene. Second, bacteria

JA200/pLC30-20 had the highest cellular level of RecA protein since they contained numerous copies of the recA gene. However, derepression of the recA gene was less dramatic than in wild type bacteria (3- versus 9-fold)(data not shown). This result suggests that there was incomplete cleavage of LexA protein in that strain, which had constitutively 11-fold more RecA protein than the wild type strain. This result may be interpreted as showing that a high cellular level of RecA protein inhibits the formation of RecA protease. Finally, the cellular level of RecA protein did not increase after UV-irradiation in bacteria bearing a lexA3 mutation (Figs 3 and 4). This agrees with the fact that LexA3 protein is resistant to cleavage by RecA protease (Little et al. 1980).

The different cellular levels of RecA protein present after 90 min of post-irradiation incubation (Fig. 4) can grossly account for the cellular resistance to UV-light (Fig.2) or MMC (Fig. 1).

That cell survival depends on the cellular level of RecA protein was strenghtened by the finding that bacteria DM2210 : lexA3 RecAo were slightly more sensitive than wild-type bacteria but much more resistant to UV-light than DM1623 : lexA3 $recA^+$ bacteria. Our results also support the idea that LexA repressor regulates genes other than recA, such as uvrA and umuC, involved in DNA repair (Kenyon and Walker 1981). The latter repair processes may account for the 1.3-fold sensitization to UV-light of lexA3 RecAo bacteria as compared to $lexA^+$ RecAo or $lexA^+$ $recA^+$ bacteria (Fig. 2).

2. An elevated cellular level of RecA protein did not affect the extent and the kinetics of UV-reactivation.

Higher survival and mutagenesis of UV-irradiated phage λ in UV-irradiated bacteria (UV-reactivation) appears to be the expression of inducible error-prone repair in bacteria (Defais et al. 1971 ; George et al. 1974).

Since the kinetics of induction of UV-reactivation (Defais et al. 1976) is similar to the kinetics of induction of RecA protein synthesis (Gudas and Pardee 1976), it was more or less admitted that UV-reactivation of phage λ was dependent upon the induced synthesis of RecA protein.

Two sets of experiments rule out this assumption.

We found that UV-irradiated phage λ survived equally well when it developed in non-irradiated bacteria such as AB1157 or DM211 : RecAo, or JA200/pLC30-20 : ColE1 recA$^+$ (Fig. 5A).Note that in the absence of a DNA damaging treatment, JA200/pLC30-20 and DM2211 bacteria had a basal level of RecA protein respectively 11 and 7.5-fold higher than that of AB1157 bacteria (Figs 3 and 4).

This result shows that in the absence of damage to host DNA, an elevated cellular level of RecA protein failed to promote a higher survival of UV-irradiated phage λ.

When host bacteria were irradiated before λ infection, the efficiency of UV-reactivation was found to be similar among various hosts AB1157, or DM2211 : RecAo, or JA200/pLC30-20 : ColE1 recA$^+$ (Fig. 5A).

We not only measured the extent of UV-reactivation but also its kinetics according to Defais et al. (1976). UV-irradiated host bacteria were incubated for various periods of time before infection with UV-irradiated phage λ. We found that repair (Fig. 6A) and mutagenesis (Fig. 6B) of UV-irradiated phage in UV-irradiated parental AB1157 bacteria increased during the early post-irradiation period and reached a maximum at 30 min and then decayed with a half-life of about 30 min. The same kinetics was observed in UV-irradiated bacteria overproducing RecA protein (DM2211 or JA200/pLC30-20) (Figs 6A and B).

We conclude that the amount of RecA protein is not limiting the process of UV-reactivation in wild-type bacteria.

3. An elevated cellular level of RecA protein did not suppress the effects of a lexA3 mutation upon UV-reactivation.

The lexA1 mutation, which prevents induction of RecA protein synthesis (Devoret et al. 1981), also inhibits UV-reactivation of UV-irradiated phage λ (Defais et al. 1971).

Since an elevated cellular level of RecA protein restored resistance to UV-light of a lexA3 mutant, we asked the question : does it also restore UV-reactivation of damaged phage λ in such a mutant ?

There was no UV-reactivation of phage λ in DM2210 : lexA3 RecAo bacteria. Phage survival remained unchanged in non-irradiated or UV-irradiated lexA3 RecAo bacteria as well as in DM1623 : lexA3 $recA^+$ bacteria (Fig. 5B). Moreover, whatever the incubation period of DM2210 or DM1623 bacteria before infection, there was no induced phage λ reactivation or mutagenesis (Fig. 6 C and D).

The fact that phage survival was similar in non-irradiated AB1157 : lexA$^+$ or DM1623 : lexA3 host bacteria (Fig. 5) rules out the possibility that the lexA3 mutation might affect phage development when the host cell was not damaged with UV-light.

4. Induction of prophage λ is not sensitized to UV-irradiation by an elevated cellular level of RecA protein.

Upon prophage λ induction, λ repressor is inactivated by proteolytic cleavage (Roberts and Roberts 1975). In vitro, RecA protein cleaves λ repressor (Roberts and Roberts 1981).

We found that spontaneous production of phage λ was similar in the two lysogens GY4453 : RecAo (λ) and GY4454 : recA$^+$ (λ) (Fig. 7) although the basal level of RecA protein was 7.5-fold higher in GY4453 than in GY4454 (Fig. 3). Our results confirm that, in the absence of DNA damage, elevated cellular levels of RecA protein fail to trigger or to sensitize UV-induction of prophage λ (McEntee 1977 ; Roberts et al. 1977 ; Ogawa et al. 1978 ; Eitner et al. 1980). In fact, prophage λ was induced by the same UV-dose given to the two lysogens (Fig. 7).

Note that in lexA3 recA$^+$ lysogenic bacteria, prophage λ was induced with about a 40 % efficiency of that of the parental bacteria (Fig. 7) even though the basal level of RecA protein was much lower in lexA3 than in the parental bacteria and was not induced by UV-irradiation (Figs. 3 and 4). This result confirms that in recA$^+$ bacteria, prophage λ induction does not require full induction of RecA protein synthesis (Baluch et al.

1980 ; Moreau et al. 1980a; Devoret et al. 1981).

5. The efficiency of prophage λ induction in a lexA3 RecAo mutant was reduced in spite of an elevated cellular level of RecA protein.

We tested whether RecA protease activity depended on the cellular level of native RecA protein. In other words, was the efficiency of prophage λ induction enhanced in a lexA3 RecAo mutant overproducing RecA protein ?

We found that prophage λ was induced with a similar UV-dose response in the two lysogenic bacteria, GY4451 : lexA3 recA$^+$ (λ), and GY4452 : lexA3 RecAo (λ). In both mutants, the efficiency of induction was 30-40 % of that of the parental lysogenic bacteria (Fig. 7). This shows that the low efficiency of prophage λ induction found in a lexA3 mutant cannot be ascribed to a low basal level of RecA protein.

DISCUSSION

1. We observed that survival of excision-proficient bacteria to UV-irradiation was related to the cellular level of RecA protein except for the double mutant lexA3 RecAo. The lexA3 mutation prevents the induced synthesis of RecA protein and renders LexA protein resistant to cleavage by RecA protease (Little et al. 1980) and the cells sensitive to UV-irradiation. The RecAo phenotype, likely due to a cis-acting mutation near or in the recA gene, produced an artificially enhanced cellular level of RecA protein that compensated much for the lack of cleavage of LexA protein.

Note that the survival to UV-light of the double mutant lexA3 RecAo was slightly more sensitive than wild type ; the dose-reduction factor was about 0.75 so that one can deduce (Devoret et al. 1975) that 25 % of the lethal lesions remaining in the DNA of a lexA3 RecAo mutant are repaired in a wild type strain by lexA-controlled functions other than induction of RecA protein synthesis.

Until now, six genes have been shown to be induced by DNA damage (Kenyon and Walker 1980) and therefore likely to be lexA-controlled genes. The expression of one of them, called uvm (Steinborn 1978) or umuC (Kato and Shinoura 1977) appears indispensable to promote an enhanced repair of UV-damaged phage λ (UV-reactivation) (Steinborn 1979 ; Quillardet and Devoret 1981).

It is our contention that the uvm function is also required for efficient cell survival. As compared to that of

the parental strain AB1157, survival to UV irradiation of the double mutant lexA3 RecAo was similar to that of uvm-25, a UV non-mutable mutant (Steinborn 1978).

2. Survival of UV-damaged phage λ is enhanced when the host-cell is irradiated (Weigle 1953), that is, when lexA-controlled functions can be induced in the host cell (UV-reactivation). When the host cell had an artificially elevated level of RecA protein, but carried a lexA3 mutation, UV-reactivation still failed to occur (this work).

The efficiency of repair of UV-damaged phage λ in the UV-irradiated uvm-25 mutant was equal to that found in an intact wild type strain but less efficient than in a UV-damaged wild type strain (Quillardet and Devoret 1981). The 25 % observed increase in the removal of lethal lesions in the phage DNA was gained by the effect of the so-called UV-reactivation process, in other words, by lexA-controlled functions other than induction of RecA protein synthesis.

In contrast to what was observed for the survival of UV-damaged bacteria, survival of phage λ to UV-irradiation was independent of the level of RecA protein in non-damaged lexA3, lexA3 RecAo, RecAo or wild type hosts and was equivalent to that observed in a UV-damaged lexA3 or uvm mutant. As long as there is a basal concentration per cell of about 4000 molecules of RecA protein, they are enough RecA molecules to help to repair phage DNA.

The different requirements in the cellular concentration of RecA protein for bacterial survival as opposed to phage survival can easily be accounted for by the fact that chromosomal

DNA is 50 times longer than phage DNA. A given UV fluence produces 50 times more lesions in the bacterial chromosome than in phage DNA. Consequently, there is a need for more molecules of RecA protein to help to repair the bacterial chromosome.

3. lexA-controlled functions other than induction of RecA protein synthesis contribute to promote efficient cell survival to UV-light. They remove 25 % of lethal lesions in bacterial DNA. They play a role in the restoration of cell survival that is more notable than claimed by some authors (Salaj-Smic et al. 1979). Furthermore, lexA-controlled functions may confer to the bacteria an increased fitness to environmental changes in switching on error-prone repair and therefore bacterial mutagenesis as suggested by the Uvm phenotype.

4. Cleavage of LexA protein is involved in various cellular functions induced by DNA damage among them is the amplification of RecA protein synthesis. It is clear that recognition of this fact provides an easy explanation for the similarity observed between the time course of induction of RecA protein synthesis (Gudas and Pardee 1976) and that of UV-reactivation (Defais et al. 1976). Induction of RecA protein synthesis and of UV-reactivation of UV-damaged phage λ were fully expressed but only after a 30 min delay ; this delay may reflect the period of time necessary to obtain extensive cleavage of LexA protein.

5. Prophage λ induction requires the formation of RecA protease that cleaves λ repressor (Roberts and Roberts

1981). Yet, there is no proportionality between the amounts of RecA protease and native RecA protein in a cell. The basal cellular level of RecA protein can give rise to enough RecA protease molecules to induce prophage λ. In lexA3 bacteria carrying either a mutation that caused overproduction of RecA protein or the recA$^+$ allele, we found that the efficiency of induction of prophage λ was 40 % of that observed in the parental bacteria. The level of RecA protein in the lexA3 recA$^+$ mutant was 80 % of the basal level in the lexA$^+$ recA$^+$ parental bacteria and was constant even after UV irradiation. These findings confirm reports from this laboratory (Moreau et al. 1980a ; Devoret et al. 1981) and from another (Baluch et al. 1980) that induction of prophage λ or 80 is independent of the induction of RecA protein synthesis.

The reduced efficiency of prophage λ induction observed in a lexA3 mutant or in a lexA1 mutant (Devoret et al. 1981), was independent of the cellular level of RecA protein. We assume that UV-reactivation acts on an induced prophage as well as on an infective phage. Upon lysogenic induction by UV light, development of the induced prophage into viable mature phage occurs under the very conditions that promote UV-reactivation of UV-damaged phage λ. Phage DNA as well as host DNA are damaged by UV light. The efficiency of prophage induction as measured by the production of infective centers depends not only on the proteolytic cleavage of λ repressor but also on induced repair of the damaged prophage DNA.

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TABLE 1. Bacterial strains

Strains	Relevant chromosomal markers			Plasmids or prophages	Origin or source
	recA	lexA	uvrA		
AB1157	+	+	+		Howard-Flanders
DM1623	+	lexA3	+		AB1157
DM2210	RecAo	lexA3	+		Ginsburg and Mount
DM2211	RecAo	+	+		"
JA200/pLC30-20	recA1	+	+	F^+/ColE1 $recA^+$	Clarke and Carbon (1976)
KM1842	ΔrecA	spr-51	+	F^+/ColE1 $recA^+$	Weinstock et al. (1979)
GY105	+	+	uvrA16		E.coli K12 hcr^- Harm (1963)
GY108	+	+	uvrA16	(λind^-)	GY105
GY4015	+	+	+		C600 Amp^R; Moreau et al. (1976)
GY4450	recA1	+	+	F^+	Plasmidless segregant of JA200/pLC30-20
GY4451	+	lexA3	+	(λ)	DM1623 (λ)
GY4452	RecAo	lexA3	+	(λ)	DM2210 (λ)
GY4453	RecAo	+	+	(λ)	DM2211 (λ)
GY4454	RecAo	+	+	(λ)	AB1157 (λ)
GY4915	ΔrecA	spr-51	+	F^+	Moreau et al.(1980)

FIGURE LEGENDS

FIGURE 1. Test of colony resistance to mitomycin C

Master plates with gridded colonies were replica-plated onto EMBO plates containing in the top soft agar increasing quantities : 0, 5, 15, 30, 40, 50 or 60 µl of a 1 µg/µl MMC solution and the replica-plates were incubated overnight at 37°C. As a function of MMC concentration the patches were :(i) pinkish white (normal growth : solid bands) ; (ii) dark purple (poor growth : striaed bands) ; (iii) absent (no growth : open bands).

FIGURE 2. Cell survival as a function of UV dose

Exponentially growing bacteria were diluted in 0.01 M $MgSO_4$ and the diluted samples were exposed to various doses of UV light indicated on the abscissa. AB1157 : *lexA*$^+$ *recA*$^+$ (•), DM1623 : *lexA3* *recA*$^+$ (Δ), GY4450 : *lexA*$^+$ *recA1* (▽), DM2211 : *lexA*$^+$ RecAo (0), DM2210 : *lexA3* RecAo (▲), JA200/pLC30-20 : *lexA*$^+$ *recA1*/ColE1 *recA*$^+$ (■).

FIGURE 3. Cellular level of RecA protein

After irradiation at the indicated doses, the cell suspensions were incubated for 40 (panel A) or 30 min (panel B) at 37°C.At time 0 (zero dose) or after irradiation, 9 ml (panel A) or 25 ml (panel B) samples were taken and processed for immunoelectrophoresis as described in Materials and Methods. In the last lane of panel B were put 5 µg of purified RecA protein.

FIGURE 4. Time course of induced RecA protein synthesis

Bacteria were treated as described in Fig. 3. AB1157 : *lexA*$^+$ *recA*$^+$ (●) ; DM1623 : *lexA3* *recA*$^+$ (△) ; DM2211 : *lexA*$^+$ RecAo (o) ; DM2210 : *lexA3* RecAo (▲) ; JA200/pLC30-20 : *lexA*$^+$ *recA1*/ColE1 *recA*$^+$ (■). The concentration of RecA protein relative to the total protein concentration is normalized to the background level observed in AB1157 : *lexA*$^+$ *recA*$^+$ and is plotted against the time of post-irradiation incubation.

FIGURE 5. UV-reactivation of phage λGY15 in *lexA*$^+$ or *lexA3* host bacteria.

Exponentially growing bacteria, at a concentration of $5x10^8$/ml, were centrifuged, resuspended in 0.01 M $MgSO_4$ and UV-irradiated at a dose giving 1 % survival. The control culture was treated as above except for UV-irradiation. Bacterial suspensions were diluted by the addition of an equal volume of double strength LB medium and incubated with agitation at 37°C. After 30 min of incubation, 0.1 ml aliquot were taken and infected with 0.1 ml samples of phage λGY15 irradiated at doses indicated on the abcissa. The multiplicity of infection was lower than 10^{-3}. After 15 min of phage adsorption, infective centers were measured. Phage survival is plotted on the ordinate. A : Phage survival on UV-irradiated (closed symbols) or non-irradiated (open symbols) *lexA*$^+$ host bacteria : AB1157 : *lexA*$^+$ *recA*$^+$ (●,o) ; DM2211 : *lexA*$^+$ RecAo (▲,△) ; JA200/pLC30-20 : *lexA*$^+$ *recA1*/ColE1 *recA*$^+$ (■,□). B : Phage survival on UV-

irradiated (closed symbols) or non-irradiated (open symbols) lexA3 host bacteria DM1623 : lexA3 recA$^+$ (▼,▽) ; DM2210 : lexA3 RecAo (▲,△).

FIGURE 6. Kinetics of survival and mutation to virulence of UV-irradiated phage λGY15.

Exponentially growing bacteria, at a concentration of $5x10^8$/ml, were centrifuged, resuspended in 0.01 M $MgSO_4$ and UV-irradiated. The control culture was treated as above except for UV-irradiation. Bacterial suspensions were diluted by the addition of an equal volume of double strength LB medium and incubated with agitation at 37°C for various periods of time. Bacterial samples were taken at the times indicated on the abcissa and infected with UV-irradiated phage λGY15 at a multiplicity of infection lower than 10^{-1}. After 15 min of phage adsorption, parental and virulent phages were assayed. The reactivation factor and the frequency of mutation to virulence are plotted on the ordinate. A : Reactivation factor of UV-irradiated phage λGY15 grown on UV-irradiated AB1157 : lexA$^+$ recA$^+$ (●) ; DM2211 : lexA$^+$ RecAo (▲) ; or JA200/pLC30-20 : lexA$^+$ recA1/ColE1 recA$^+$ (■). B : Frequency of mutation to virulence of UV-irradiated phage λGY15 grown on UV-irradiated (closed symbols) or non-irradiated (open symbols) : AB1157 : lexA$^+$ recA$^+$ (●,○) ; DM2211 : lexA$^+$ RecAo (▲,△) ; or JA200/pLC30-20 lexA$^+$ recA1/ColE1 recA$^+$ (■,□). C : Reactivation factor of UV-irradiated phage λGY15 grown on UV-irradiated DM1623 : lexA3 recA$^+$ (▼) or DM2210 : lexA3 RecAo (▲). Frequency of mutation to virulence of UV-irradiated phage λGY15 grown on UV-irradiated

(closed symbols) or non-irradiated (open symbols) : DM1623 : lexA3 recA$^+$ (▼,▽) or DM2210 : lexA3 RecAo (▲,△).

FIGURE 7. Induction of prophage λ by UV-light

Exponentially growing bacteria, at a concentration of 10^8/ml, were centrifuged, resuspended and serially diluted in 0.01 M $MgSO_4$. Dilutions were exposed to various doses of UV-indicated on the abcissa. All samples were assayed for viable cells and infective centers. The number per ml of surviving colonies (open symbols) or induced infective centers (closed symbols) formed by GY4451 : lexA3 recA$^+$ (λ) (▲,△), GY4452 : lexA3 RecAo (λ) (▽,▼), GY4453 : lexA$^+$ RecAo (λ) (□,■), GY4454 : lexA$^+$recA$^+$ (λ) (o,●), are represented on panel A. The efficiency of induction of prophage λ, shown on a metric scale in panel B, was calculated form the data of panel A ; it corresponds to the percentage of bacteria GY4451 : lexA3 recA$^+$ (λ) (▲), GY4452 : lexA3 recA$^+$(λ)(▼),GY4453 : lexA$^+$ RecAo (λ) (■), or GY4454 : lexA$^+$recA$^+$(λ) (●) giving rise to an infective center.

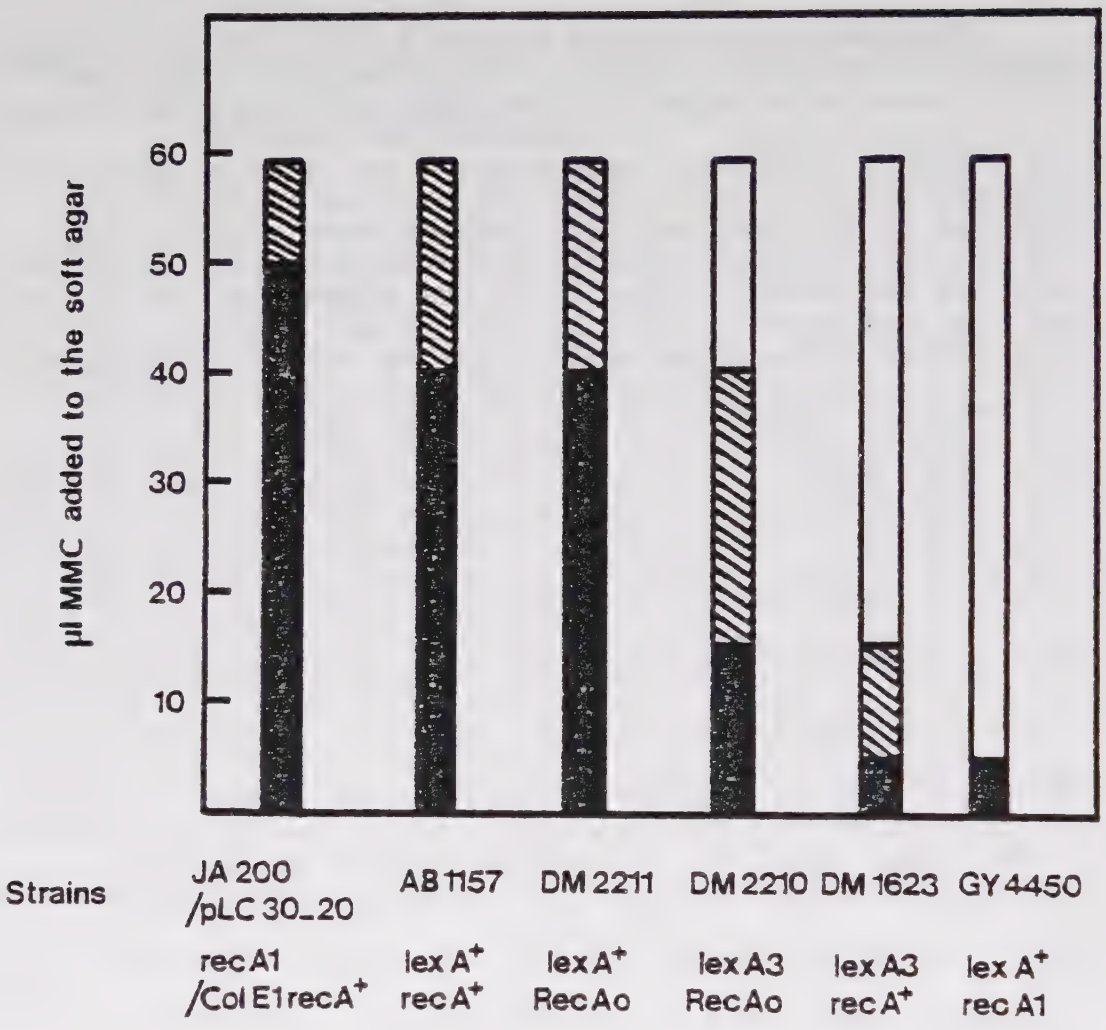
µl MMC added to the soft agar
60
50
40
30
20
10
Strains
JA 200 /pLC 30_20
recA1 /Col E1 recA+
AB 1157
lex A+ rec A+
DM 2211
lex A+ Rec Ao
DM 2210
lex A3 Rec Ao
DM 1623
lex A3 rec A+
GY 4450
lex A+ rec A1

FIG. 1

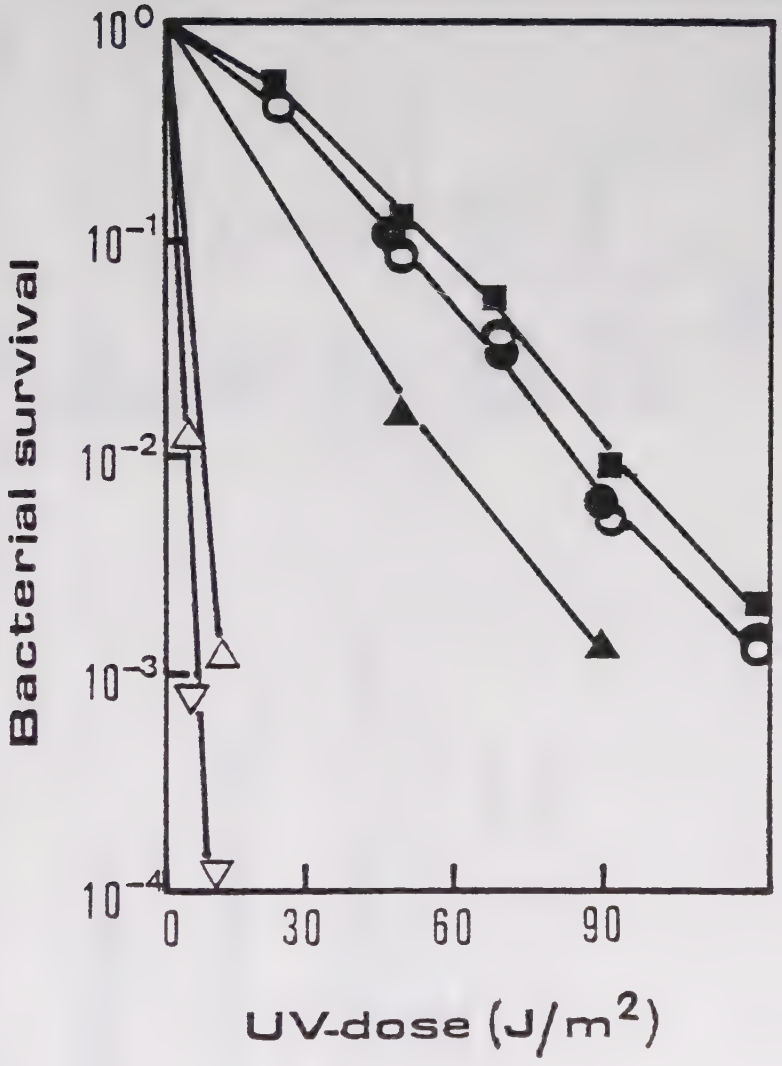

FIG.2

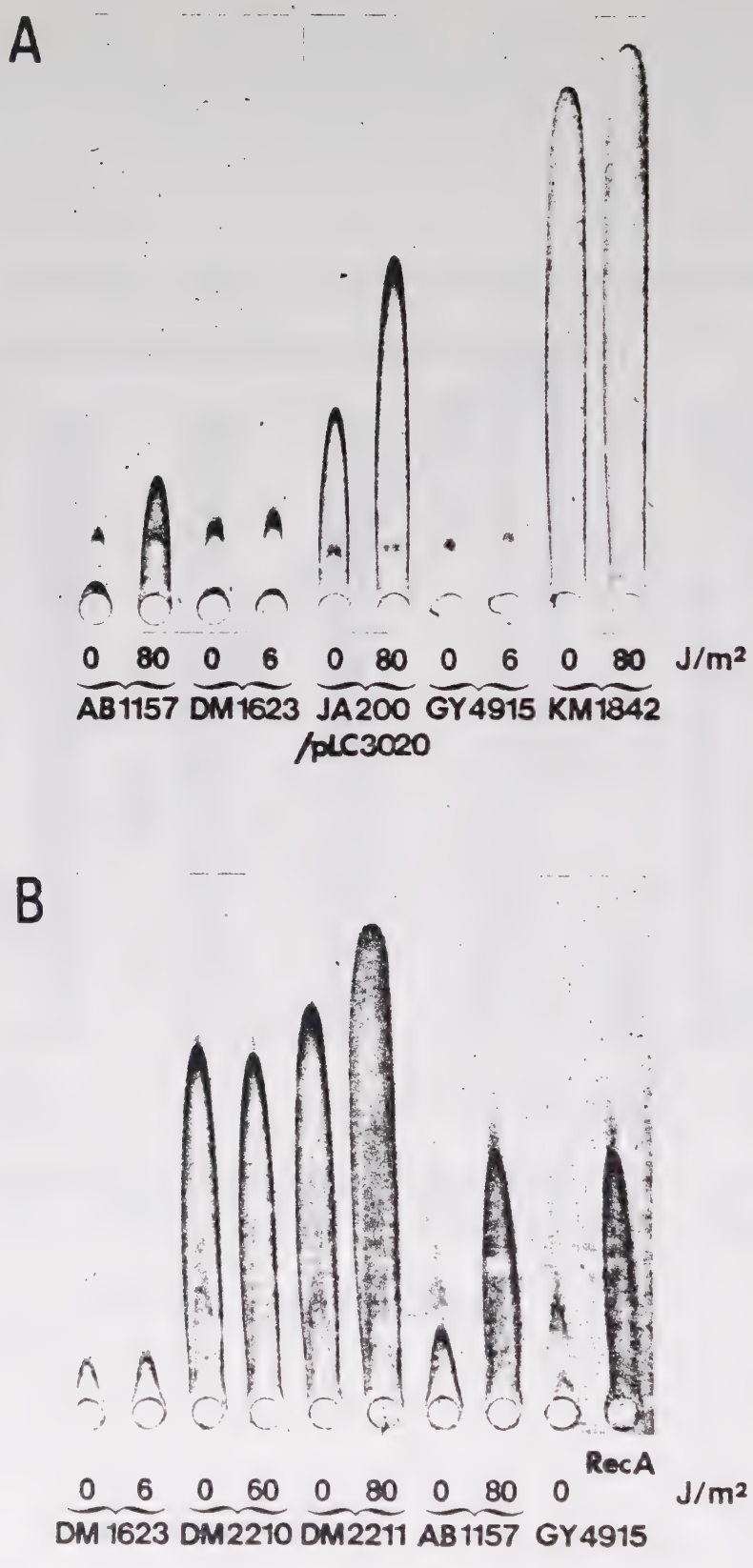
A
0 80 0 6 0 80 0 6 0 80 J/m²
AB1157 DM1623 JA200 /pLC3020 GY4915 KM1842
B
RecA
0 6 0 60 0 80 0 80 0 J/m²
DM 1623 DM2210 DM2211 AB1157 GY4915

FIG. 3

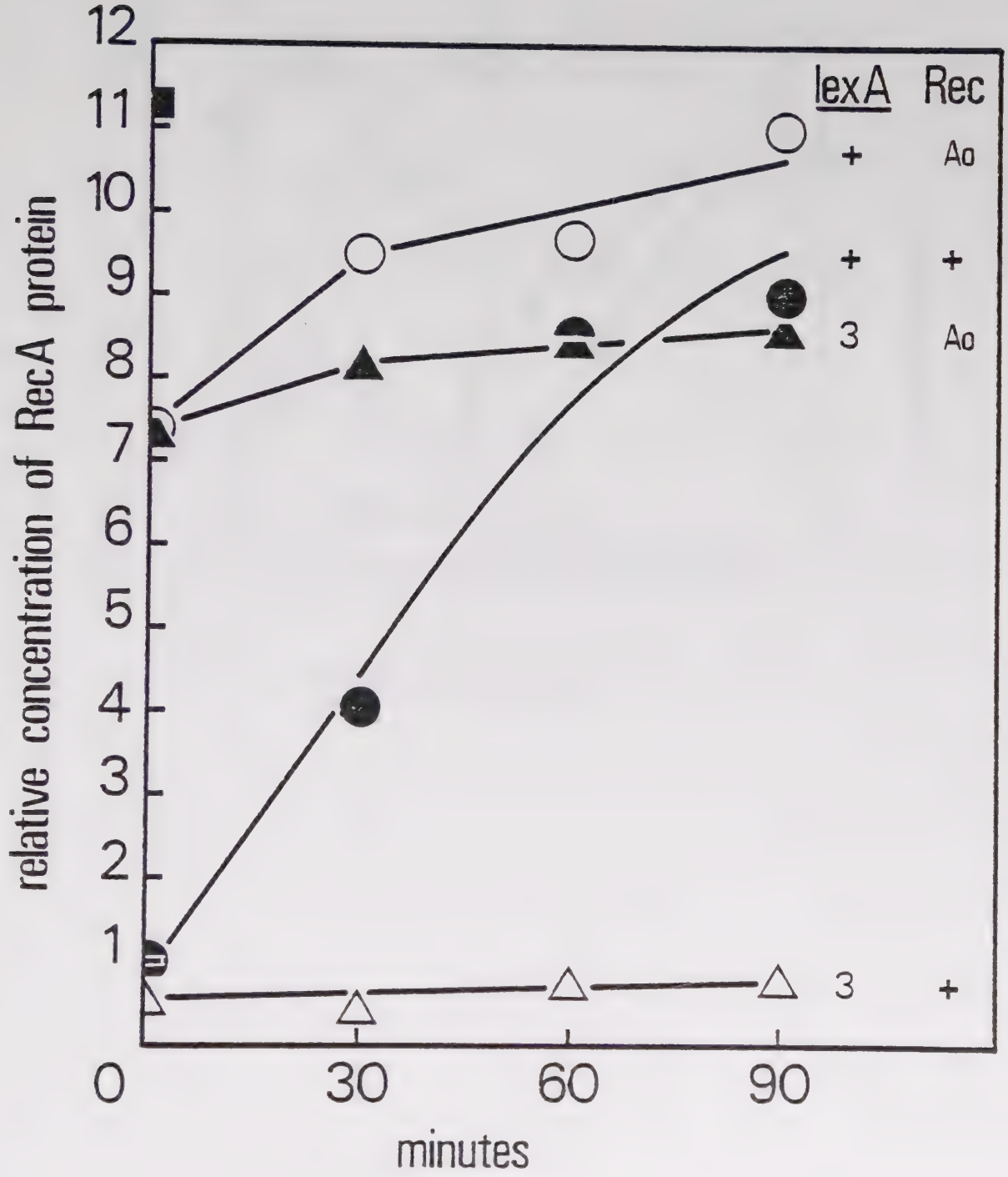
relative concentration of RecA protein
12
11
10
9
8
7
6
5
4
3
2
1
0
30
60
90
minutes
lexA
Rec
+
Ao
+
+
3
Ao
3
+

FIG.1

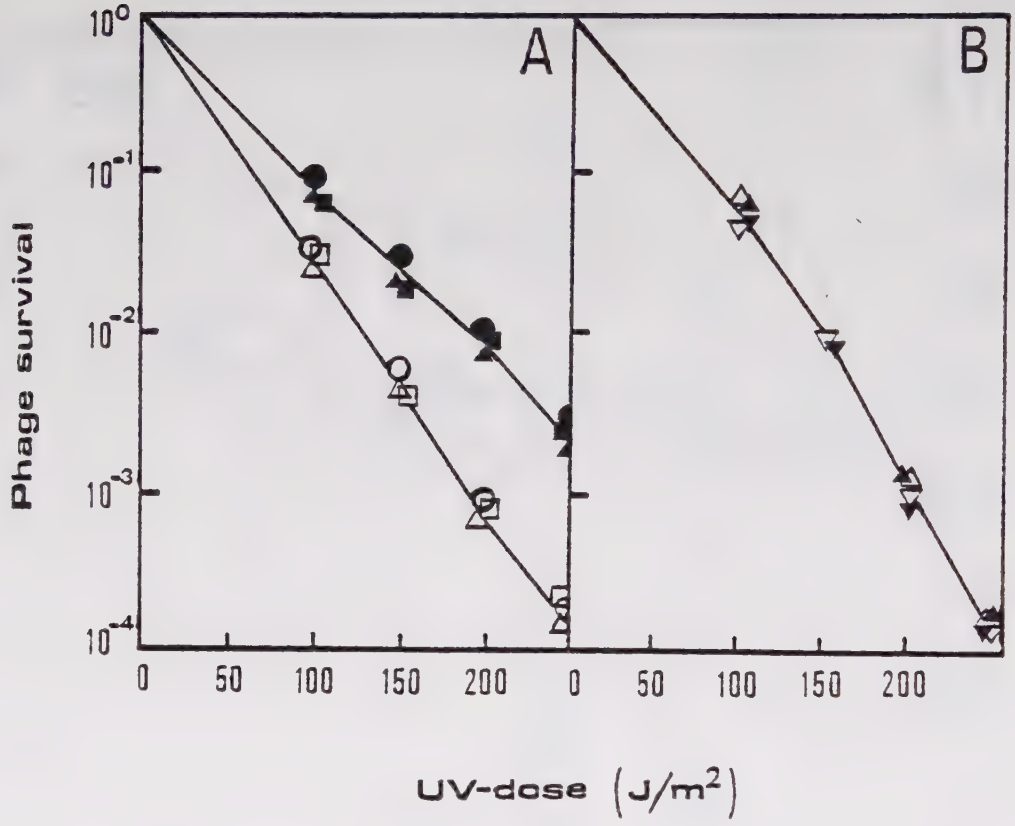
A
B
Phage survival
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
50
100
150
200
UV-dose (J/m^2)

FIG.5

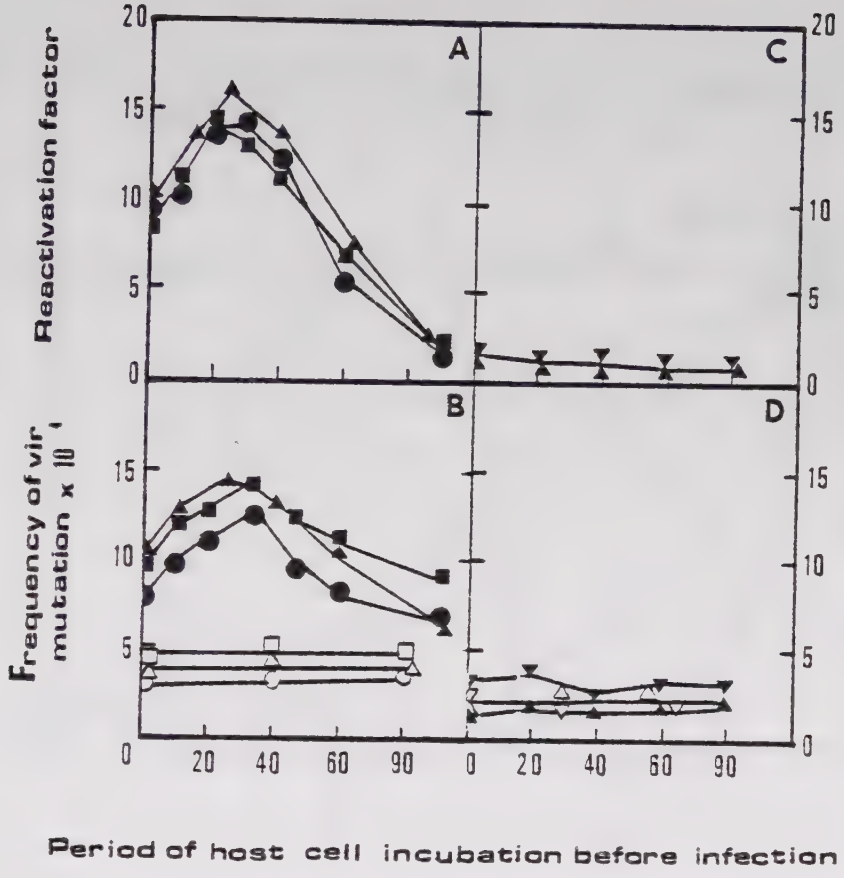
Reactivation factor
Frequency of vir
mutation x 10
A
B
C
D
0
5
10
15
20
20
40
60
90
Period of host cell incubation before infection

FIG.6

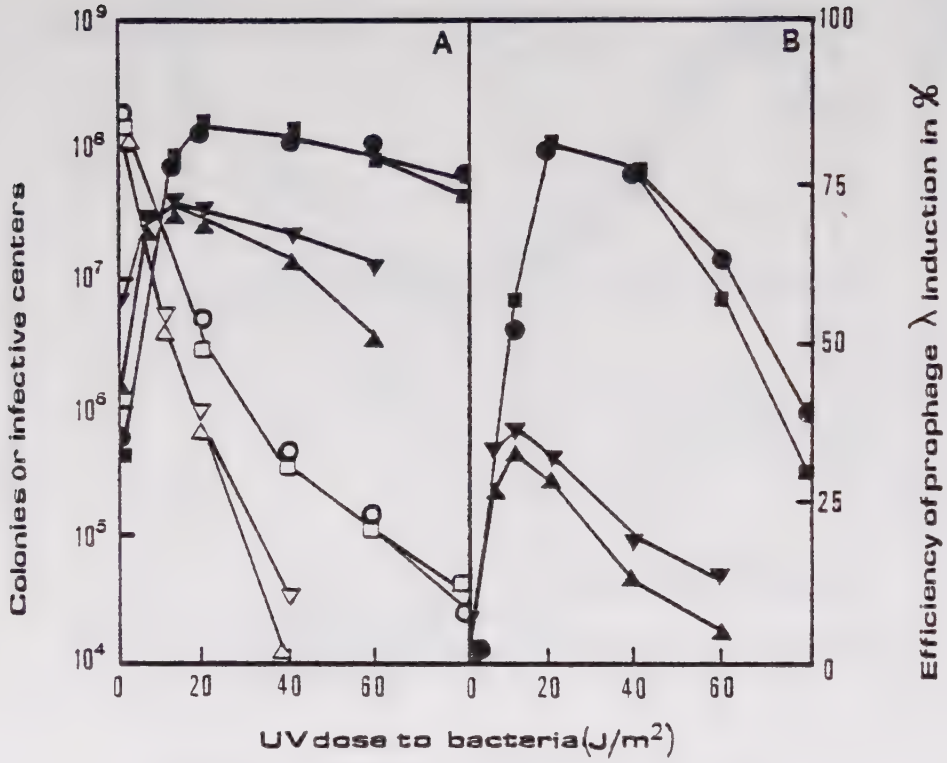
A
B
Colonies or infective centers
Efficiency of prophage λ induction in %
UV dose to bacteria(J/m²)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
100
75
50
25
0
0
20
40
60

FIG.7

INDUCTION OF PROPHAGE 80 IN A *recA* MUTANT OF *Escherichia coli* K12

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Running head:Prophage 80 induction in a *recA430* mutant

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ABSTRACT

Prophage 80 was found to be induced by ultraviolet (UV) light and thymine deprivation in *recA430* (previously called *lexB30*) mutants in which prophage λ is not inducible whereas recombination is only slightly altered. Yet, prophage 80 was not induced in two recombination-deficient *recA13* and *recA99* lysogens. A mutant of prophage 80 carrying an *ind*$^-$ mutation was not induced either.

Exposure to UV light of *recA430* mutants lysogenic for 21 or 434, two other lambdoid prophages, showed that prophage 21 was induced up to 1% whereas prophage 434 produced less than 10^{-6} clear-plaques.

In a *recA430* mutant, after 30 min of incubation, the synthesis of RecA protein was induced to about 40% of that observed in the *recA*$^+$ control. Since prophage 80 repressor is likely to be inactivated by RecA protease as are λ repressor and LexA protein, our data suggest that RecA430 protein still possesses sufficient proteolytic activity to cleave 80 repressor fully but not LexA protein that was subjected to limited cleavage. Full induction of prophage 80 was also observed in *lexA1 recA430* and *lexA1 recA*$^+$ in which RecA protein synthesis failed to be induced.

The prophage and LexA repressors studied here display a decreased susceptibility to RecA430 protease in the order: 80> LexA> 21> λ ⩾ 434. The differential efficacy of RecA430 protease may be accounted for by: (i) the alteration of RecA430 protein that may extend beyond the proteolytic domain and affect RecA protein binding to repressor molecules (ii) the in vivo equilibrium between cleavable (monomeric) and non-cleavable (dimeric) repressor forms that may vary among lambdoid phages.

Our results provide some molecular insight to account for the fact that the time sequence of the various SOS functions induced by DNA damage is in part regulated by the differential susceptibility of prophage and LexA repressors to wild type RecA protease.

INTRODUCTION

Phage λ can reside in its host Escherichia coli K12 in a dormant state as prophage. Other temperate prophages such as 434, 21, or 80 share many properties with prophage λ so that they have been designated lambdoid prophages.

All lambdoid prophages are induced to produce progeny phage in response to agents or treatments that damage the DNA of the lysogenic host (see recent reviews by Witkin 1976 and by Devoret 1981). Jacob and Monod (1961) postulated that a common cellular inducer inactivates the repressors of lambdoid prophages.

RecA protein inactivates λ and P22 repressors by proteolytic cleavage in vivo and in vitro (Roberts and Roberts 1975; Craig and Roberts 1980; Roberts, Roberts, Craig and Phizicky 1978; Phizicky and Roberts, 1980) and appears, therefore, to be the inducer of inducible prophages. RecA protease also cleaves LexA protein (Little et al. 1980), the repressor of recA and lexA genes (Little et al. 1981; Brent and Ptashne 1981). LexA protein appears to repress genes that govern various functions induced by DNA damage (SOS functions) such as inducible error-prone repair and cell filamentation (Kenyon and Walker 1980).

Mutants devoid of RecA protein (McEntee 1977) have a complex phenotype, similar to that displayed by missense or amber recA⁻ mutants (Mount 1971): they are deficient in genetic recombination, in diverse repair pathways and in prophage induction.

Mutations in the recA gene that would affect rather specifically the proteolytic activity of RecA protein might help to define precisely the requirement for RecA protease activity in RecA-dependent phenomena such as

mutagenesis by ultraviolet light, prophage induction, and general genetic recombination. In other words, how much are all activities of RecA protein promoted by its proteolytic action?

Bacterial mutants specifically deficient in prophage λ induction were isolated (Devoret and Blanco 1970). One of them called lexB30 (Blanco, Lévine and Devoret 1975) prevents induction of prophage λ whereas it does not affect much recombination proficiency (Morand et al. 1977a and 1977b). It has now been renamed recA430 since it maps in the recA gène as evidenced by biochemical tests (McEntee 1977; Roberts and Roberts 1981), and, as demonstrated by genetic tests (Morand et al. 1977b; Castellazzi et al. 1977), it defines a cluster of mutations close to the recA promoter (Sancar and Rupp 1979) which affect RecA-promoted inducible functions.

We found that, in sheer contrast to prophage λ, prophage 80 was fully induced in recA430 lysogens. We then determined the in vivo effects of the recA430 mutation on RecA430 protease activity against repressors estimated by the pattern of induction by UV-light of prophages 80, 434, and 21. Measurements of the rate and of the amount of RecA430 protein synthesized after UV-irradiation permitted us to monitor also indirectly the efficiency of cleavage of LexA protein by RecA430 protease.

MATERIALS AND METHODS

1- Bacterial strains

a) The mutation called previously lexB30, shown to be located in the recA gene (Morand et al. 1977a; Morand et al. 1977b; Castellazzi et al. 1977), has been renamed recA430, in order to conform with the established genetic nomenclature.

From the original strain GY1163, the recA430 mutation was transferred to strains AB1157 (Castellazzi, pers. comm.) and AT713 (Morand et al. 1977a) by phage P1 transduction, and from GY1139 to GY3450 by chromosomal mobilization with F'15 and recombination (Blanco, pers. comm.). The background markers carried by the three recipient strains are for JM776: thr-1 leu-6 thi-1 proA2 his-4 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 str-31 tsx-33 sup-37; for GY3450: thyA301 his-108 bio-87 metS120 ilvA106 pheA97 str-8 endA101; and for AT713: thi-1 lysA22 argA21 cysC43 malA1 xyl-7 mtl-2 lam str-104 rel-1. The use of three genetic backgrounds permitted us to ascertain the phenotypic specificity conferred by the recA430 mutation.

Since many recA mutations such as recA13 are missense mutations and therefore might produce altered RecA protein, we also used a strain carrying an amber mutation recA99am (Mount 1971).

The strains used are listed in table 1; many of them are lysogenic for various prophages described in table 2.

b) In order to use the ampicillin method for the detection of infective centers formed by either prophage 80 or prophage λ, we constructed the indicator strain GY4925:Amp^R $TonA^+$ by derivation from GY4015 (Moreau et al. 1976), an ampicillin resistant derivative of C600. The tonA mutation present in GY4015 that renders the strain resistant to phage 80 was crossed

out by conjugation with Hfr P4X6 and selection for Thr^+ Leu^+ Amp^R recombinants which had acquired the $TonA^+$ marker.

c) Since infective centers revealed with the use of the ampicillin method are rather clear, we also employed GY105 as indicator strain without ampicillin to determine the relative proportion of turbid and clear plaques.

d) Unless otherwise stated all cultures were incubated at 37°.

2- Phages

a) The phages used are indicated in Table 2. There is still no satisfactory designation of phage strains, we have been using for many years a nomenclature similar in its principle to that of R. A. Weisberg.

Except for phage F81, phage λ stocks were prepared by lysis of infected cultures. Stocks of phage 80 were made as described for phage BC3.

b) Construction of phage BC3:80ind-3

Mount (1976) showed that most λ or 434 phages isolated as turbid plaque formers at 40° on a layer of strain spr-51 recA441 (tif-1) sfiA11 were not inducible by UV-light (Mount 1977). Using this method, R. Brent isolated three non-inducible mutants from phage 80supF sus2 (Andoh and Ozeki 1968) that he kindly gave us. We called them BG8:80ind-2, BG9:80ind-3, BG10:80ind-4.

We constructed three non-inducible 80 phages that lysogenized bacteria at att80. The supF sus2 region was crossed out by prophage reactivation and replaced by the att80 h80 region of phage F82 .

In practice, GY4830, a strain lysogenic for prophage F82:immλ att80 h80, was infected with phage BG8 or BG9 or BG10, which had been irradiated with 50 J/m^2 of ultraviolet light to enhance recombination. The infected

hetero-immune lysogens, diluted a 100-fold, were incubated for two hours to allow cell lysis and the release of recombinant phages.

One ml of this phage lysate was then added to a 0.25 ml suspension of strain T5 lysogenic for λt5 (Eisen et al. 1966), an O⁻ defective prophage. The use of strain T5 permitted us to collect recombinant phages carrying the att80 h80 regions and to eliminate the λcI recombinant and parental phages. Phages 80att80 h80 were selected as 80 immune lysogens after plating 0.1 ml of the T5 cell suspension on EMB0 plates with 3 ml of soft agar containing 0.1 ml of phage BD1:80cI⁻ at 10^{10}/ml. GY4927 was one of the 80 lysogens having the lowest spontaneous production of free phage, it carries prophage BC3:ind-3.

c) Preparation of stocks of phage BC3:80ind-3

BC3:80ind-3 stocks had to be produced by zygotic induction to avoid the construction of lysogens with 80ind⁺ phage revertants. Zygotic induction was promoted by F⁺ chromosome mobilization. GY4927 was made F⁺ in two steps:(i) we isolated GY4938, a mal⁻ derivative of GY4927, by selecting white colonies with phage E50 on EMB-Mal medium, (ii) GY4938 was crossed with W1485:F⁺ and the transfer of the F sex factor was detected by the pinkish color of the F⁺ colonies on EMB-Mal plates, one of which gave GY4943:F⁺ (BC3).

Stocks of BC3 were made as follows: GY4943 was crossed with C600 for 140 minutes; 0.1 ml samples of the mating mixture were plated with GY4925 indicator bacteria in the soft agar. The following day, the soft agar was then scraped out of the plates, crushed in a centrifuge tube, and the debris spun down. The supernatant contained usually 10^{11} phage/ml.

3- Media and chemicals

LBTU contained 10 g of NaCl, 10 g of tryptone Difco, 5 g of yeast extract, and 40 mg of thymine and of uracile per liter of demineralized water and was adjusted to pH=7 with NaOH. LAT contained 15 g of Biomar agar, 40 mg of thymine per liter of LB. These media were used as rich liquid and solid media.

GT-amp contained 5 g of NaCl, 5 g of tryptone (Difco), 8 g of peptone, and 12 g of Biomar agar per liter of demineralized water and adjusted to pH= 7 with NaOH and before pouring supplemented with 10 µg/ml of Totapen (Bristol). This medium was used for measuring induced infective centers.

Soft agar contained 7.5 g of agar Difco per liter of demineralized water.

Tris 80: Tris.HCl 6mM, pH 7.5 containing NaCl 68mM, $CaCl_2$ 0.1mM and gelatin 0.005 % was used as dilution medium for phage 80 or 80 lysogens.

EMBO contained 5 g of NaCl, 12.5 g of EMB broth base Difco, 1 g of yeast extract, and 13.5 g of Biomar agar per liter of demineralized water. When necessary it was supplemented with 2 g/l of sugar.

4- Construction of lysogens

Suspensions of bacteria to be lysogenised were infected at low multiplicity by phages carrying respectively 80 or λ immunity such as BA1 or A2, F74, F82. Bacteria at a concentration of $3x10^8$/ml, were infected with 0.001 phage per bacterium and the suspensions were incubated for 3 hours, diluted and plated on EMBO plates containing phages such as BD1 or C21 or F81 at $2x10^9$ p.f.u./ml in the soft agar. The lysogens were selected as white immune colonies.

Lysogens for 434 and 21 were isolated as purified single colonies starting from spots of confluent growth made on layers of the strains to be

lysogenized. Phages AB2 and AC3 with 434 immunity or with phage FF1 of 21 immunity were isolated in this way and their respective immunities were checked by cross-streaking them with AA2 and FF2 phages.

5- Measurement of infective centers

a) Prophage 80 infective centers were determined by plating diluted samples on GT-amp plates with 3 ml of soft agar containing either 0.05 ml of exponentially growing or 0.25ml of resting indicator bacteria GY4925. Plates were incubated at 36° when plaques formed by phage 80 were measured.

b) The efficiency of lysogenic induction was calculated as the ratio of the number of infective centers to the number of original viable lysogens.

c) Iwo et al. (1967) showed by microscopy that 80 lysogens, which had divided after the inducing treatment, could lyse and release phage. They claimed that induction of prophage 80 could be delayed by about a generation time. In order to avoid any possible overestimation of the efficiency of prophage 80 induction due to delayed induction, we used the ampicillin technique (Moreau et al. 1976) to measure infective centers formed by phage released before multiplication of the induced lysogens. We also measured infective centers formed by prophage 80 without ampicillin. The values obtained were similar.

We also determined the one-step growth curve of prophage 80 induced by UV light in GY4910:recA430 (80) and in GY4939:recA$^+$ (80) (Fig. 1). In a recA$^+$ strain, the latent period of induced phage 80 was about 105 min and a progeny of 300 phage particules was released. The recA430 mutation reduced 10-fold the size of the progeny phage. Exposure of a recA430 (80) lysogen to UV-light affected the cell capacity to produce phage.

We ascribe the results of Iwo et al. (1967) to the extended latent period of induced prophage 80, which is twice as long as that of prophage λ (60 min) (Marcovich 1956; Bailone et al. 1979).

6- Thymineless induction

The techniques used for thymineless induction were as described in Devoret and Blanco (1970).

7- UV-irradiation

Bacteria in the exponential phase of growth in LBTU medium were harvested by centrifugation and resuspended in 0.01 M $MgSO_4$ or in Tris 80, and diluted samples were exposed to UV light emitted by a 15 W General Electric germicidal lamp. UV doses were measured with a Latarjet dosimeter.

8- Immunological measurements of RecA protein

a) Cellular level.

The bacterial cultures were grown up to an A_{650} = 0.3 in enriched YM9 medium (Moreau et al.1980a) containing LB 2% plus 20 µg/ml cysteine and 10 µg/ml methionine. A sample was removed from each culture before UV-irradiation (zero dose) and processed for immunoelectrophoresis as described below. The rest of the culture was centrifuged, the pellet was suspended in cold $MgSO_4$, the suspension was then irradiated, recentrifuged and the pellet resuspended in prewarmed growth medium and further incubated with vigorous agitation. Incubation was stopped by adding cold YM9 containing 10mM NaN_3. The crude extracts were prepared as described by Roberts and Roberts (1975). Then 10 µl aliquots of the crude extracts were put into the wells of a horizontal 1 % agarose gel containing 5 % anti-RecA

serum (Laurell 1966). Electrophoresis was done at 80V for 18 h. The immunoprecipitates were stained with Amido-Black.

b) Rate of synthesis.

It was measured as previously described (Moreau et al. 1980a) as modified in Moreau et al. (1981).

9-Cleavage of λ repressor

It was measured according to Moreau et al. (1980b).

RESULTS

1- High spontaneous production and induction of prophage 80 in recA430 hosts

Bacterial mutants carrying the recA430 mutation retain recombination-proficiency whereas induction of prophage λ fails to occur (Morand et al. 1977a; Roberts and Roberts, 1981). The failure of recA430 (λ) lysogens to be inducible was thought to extend to all lambdoid lysogens, that is, to bacteria lysogenic for other lambdoid prophages. In the course of experiments on the mechanism of indirect induction (Devoret et al. 1981), we found that prophage 80 was induced by UV-light in a recA430 (80) lysogen. This finding prompted us to determine the induction patterns of lambdoid prophages in recA430 lysogens.

To ascertain that the recA430 mutation alone permitted induction of prophage 80, we constructed recA430 (80) lysogens derived from the three genetic backgrounds of JM776, GY3239 and GY3448.

All three 80 lysogens were induced by UV-light with variable efficiencies according to the genetic backgrounds of the strains used. Induction efficiency was 100% for GY4910, 40% for GY4896, and 15% for GY4911 after exposure to doses as low as 2 J/m^2 of UV light (Fig. 2). Such a dose was insufficient to induce GY4939, the parental recA$^+$ (80) lysogen, which produced 100% infective centers at a higher dose (20 J/m^2) (Fig. 3). The efficiency of thymineless induction of prophage 80 (8%) in GY4911 was half of that obtained with UV-light (curve not shown).

Spontaneous production of phage 80 was proportional to the efficiency of prophage induction by UV light (Fig. 2) from 4×10^{-2} to 8×10^{-4} in the recA430 lysogens GY4910, GY4896 and GY4911 and 4×10^{-3} in the rec$^+$ lysogen

GY4939. All infective centers were formed of turbid plaques (Table 3).

Contrasting with that of phage 80, spontaneous production of phage λ in the recA430 lysogens GY4909: (λ), GY3255: (λ) or GY5222: (F74) all carrying λcI$^+$ prophages was about 5×10^{-7} and consisted of clear plaques (Table 3). Furthermore, there was no prophage induction by UV-light in GY4909, GY3235 and GY5222 (Fig. 2), the number of infective centers did not rise over the spontaneous level as a function of the UV dose and the plaques formed were mostly clear (Table 3).

The last fact confirms that, since there is no inactivation of phage λ repressor in a recA430 mutant, spontaneous or "induced" phage production may result from the occurrence of a mutation in the cI gene that controls λ repressor synthesis.

2- Prophage 80 was not induced in a recA13 or recA99 host

The preceding results prompted us to ascertain that induction of prophage 80 in a recA430 lysogen was RecA-dependent as it is for other lambdoid prophages.

There was no induction of prophage 80 with UV-light when we tested strains GY2375: (BA1) and GY4912: (BA1), two recombination-deficient mutants carrying respectively the recA13 (missense) and recA99 (amber) mutations. The frequency of phage 80 produced spontaneously or after UV irradiation was below 10^{-5} and most of the plaques formed were clear as observed with the lysogens GY1049:recA13(λ) and GY4913:recA99(λ) (Table 3).

3- Prophage 80ind-3 was not induced in a recA430 mutant

The cIind$^-$ mutation isolated by Jacob and Campbell (1959) renders prophage λ resistant to cleavage following an inducing treatment of the

lysogen (Roberts and Roberts, 1975; Moreau et al. 1980b). Other non-inducible mutants of lambdoid phages have been isolated (Eshima et al. 1972; Mount 1976; Kirpichnikova et al. 1977; Brent, pers. commm.). In the absence of direct evidence showing that 80 repressor is cleaved by RecA protease, proof of the existence of non-inducible mutants of phage 80 would provide indirect evidence that wild type 80 repressor, like λ repressor, is subject to inactivation following inducing treatments.

The induction pattern of prophage BC3:80*ind-3* was determined first in GY4930:*rec*$^+$ (Fig. 3). The aspect of the plaques was also examined on GT plates without ampicillin: the spontaneous production of phage 80 was 7×10^{-6} and about 80% of the plaques were clear; after UV-irradiation, the number of infective centers reached 4×10^{-4} and about 70% of the plaques were clear (Table 3). In the last instance, the 100-fold increase in the number of infective centers formed (Fig. 3) can be attributed to mutation induction. Phage BC3 appears to be a bona fide 80 non-inducible mutant.

We lysogenized the *recA430* mutants JM776 and GY3448 with prophage BC3:80*ind-3* to obtain strains GY4931 and GY4944 that were subsequently exposed to UV-light. GY4931 lysogens displayed a level of spontaneous production of phage (10^{-5}) as low as that observed in *recA99* lysogenic for wild type 80. After exposure to UV-light, there was at most a 1.5 increase over the background level (Fig. 2). Similar results were observed with GY4944; this confirms that the *recA430* mutant is essentially non-mutable by UV-irradiation (data not shown).

4- Prophages 80 and λ were induced in *lexA1* lysogens in the absence of induction of RecA protein

McEntee (1977) reported that RecA430 protein was poorly induced after

infection of UV-irradiated cells by a λ transducing phage carrying the recA430 mutation. This result suggested that RecA430 protein was unable to cleave LexA protein, the repressor of the recA gene, and, therefore, prevented induced synthesis of RecA430 protein. The question of whether or not induced synthesis of RecA protein is required for lysogenic induction has a theoretical importance. If there is no requirement for induced synthesis of RecA protein, this means that the background RecA molecules present in a cell are sufficient to generate enough RecA protease.

To determine whether induction of prophage 80 occurred in the absence of induction of RecA protein, we wanted to correlate the induction patterns of prophages 80 and λ in lysogens carrying recA430 or lexA1 or both mutations with measurements of induced synthesis of RecA or RecA430 protein. The lexA1 mutation (Howard-Flanders and Boyce 1966; Mount and Kosel 1973) is similar to lexA3 (Mount et al. 1972) in that they both render LexA protein insensitive to cleavage by RecA protein (Little et al 1981) and, consequently, prevent induced synthesis of RecA protein.

Exposure to a UV-dose of 4 J/m^2 produced full induction of prophage 80 in lexA1 recA430, lexA1 $recA^+$ and $lexA^+$ recA430 mutants as it did in the parental $lexA^+$ $recA^+$ lysogen (Fig. 4).

Induced synthesis of RecA or RecA430 protein was determined in two ways: (i) by the rate of synthesis of RecA protein (Fig. 6) (ii) by the cellular level observed after a 30 min period of incubation (Fig. 7). Both procedures provided evidence that RecA protein synthesis was slightly induced in the recA430 mutant. After 30 min of incubation, the cell concentration of RecA protein relative to that of total proteins in lexA1 recA430, lexA1 $recA^+$, $lexA^+$ recA430 mutants and parental strain $lexA^+$ $recA^+$ increased respectively 0.9, 0.9, 1.7, and 4 times over the background value

(Fig. 7). The two last values indicate that, at variance with McEntee's observations, RecA430 protein, unable to cleave λ repressor in vitro (Roberts and Roberts 1981), can substantially cleave LexA protein and then allow induced synthesis of RecA430 protein.

Note that prophage λ was induced in the lexA1 recA$^+$ mutant with an 80% efficiency almost as high as that observed with prophage 80. This was corroborated by the demonstration that λ repressor was cleaved in a lexA1 mutant (data not shown) in which RecA protein was not induced. The present data confirm our previous finding that cleavage of prophage λ repressor does not require full induction of RecA protein synthesis (Moreau et al. 1980a).

All our data provide strong evidence that induced synthesis of RecA protein is not required for prophage induction of either prophage 80 or λ.

5- Different induction patterns of prophage 21 and 434 in a recA430 mutant

Since we observed opposite patterns of induction by UV light of prophages λ and 80, we wanted to determine whether other lambdoid phages might display an intermediate pattern between those of prophages λ and 80.

We tested prophage 21, whose repressor is similar to that of prophage P22 (Ballivet and Eisen, 1978). Phizicky and Roberts (1978) showed that RecA protein cleaves P22 repressor. Spontaneous production of phage 21 was almost as low as that of prophage λ and constituted of turbid plaques. After UV irradiation, 1% of the recA430 lysogens gave rise to infective centers (Fig. 5). Note that 21 behaved differently from prophage BC3:80ind-3 since all the plaques formed by 21 were turbid (Table 3). Indeed, prophage 21 seems to display a pattern of induction intermediate between prophages λ and 80.

We observed no difference between prophage λ and 434 in their induction pattern (Fig. 5 and Table 3). This confirms their close kinship.

DISCUSSION

Prophage 80 was found to be fully inducible in recA430 lysogens as evidenced by the formation of infective centers, whereas prophage λ is not (Morand et al 1977a). The contrasting effects of the recA430 bacterial mutation on the induction of prophages 80 and λ do not necessarily imply that prophage 80 is induced by a biochemical pathway different from that of prophage λ, which would be independent of the action of RecA protease. Repressors of lambdoid prophages may display a variable susceptibility to RecA protease action (Melechen and Go 1980).

Many arguments support the unitary hypothesis (Jacob and Monod 1961) that RecA protease is the common inducer of all lambdoid prophages. Three of them can be deduced from our results.

Firstly, we did not find any induction of prophage 80 in recA13 or recA99 lysogens; no induction of any lambdoid prophage has ever been reported in these mutants. This shows that when recA13 or recA99 mutations alter RecA protein more thoroughly than does a recA430 mutation, the loss of recombination proficiency also entails lack of induction of lambdoid prophages. Induction of prophage 80 as that of any other lambdoid prophage is highly dependent on RecA protein activity.

Secondly, RecA protease cleaves λ as well as P22 repressor. The two phage repressors are respectively found in Escherichia coli and Salmonella typhimurium, two different strains among Enterobacteriaceae. As prophage 80 appears more related to phage λ than is phage P22 (Botstein and Herskowitz 1974; Davidson and Szybalsky 1971; Fiandt et al 1971), it is likely that prophage 80 is induced by the very mechanism that induces other lambdoid prophages. This is all the more certain as DNA sequencing of phage 80

reveals that the cI$^+$ gene of phage 80 looks closely related to that of phage λ (Hobom, personal comm.)

Thirdly, we observed that RecA430 protein was induced to reach a 40% level of that of RecA protein after 30 min of incubation. This indicates that LexA protein, the repressor of the recA gene (Little et al 1981; Brent and Ptashne 1981), which is cleaved by RecA protein (Little et al 1981), is also cleaved by RecA430 protein although to a lesser degree.

If RecA430 protein cleaves 80 repressor, the question arises: is it the observed induced accumulation of RecA430 protein that makes the cleavage reaction possible? Three experimental facts rule out that induced synthesis of RecA protein is necessary for lysogenic induction to occur.

Firstly, the lexA1 mutation, which renders LexA protein insensitive to RecA protease and thus a stable repressor of the recA gene, prevented induced synthesis of RecA protein. In spite of this, induction of prophage 80 as well as prophage λ occurred as measured by (i) the induction of infective centers by UV-light and (ii) the actual cleavage of λ repressor. These observations are in agrement with Takebe et al (1967), who showed that prophage 80 was induced by UV light in lexA101 mutants of E. coli B. Note that induction of prophage λ was also observed in lexA3 mutants (Sedgwick et al 1981; Quillardet et al 1981).

Secondly, in the double mutant lexA1 recA430, induction of prophage 80 occurred in the absence of any induced synthesis of RecA430 protein.

Thirdly, when induced synthesis of RecA protein was reduced 3-fold by treatment of λ lysogens with rifampicin (Moreau et al 1980a), λ repressor was normally cleaved after mitomycin C treament.

Since induced synthesis of RecA protein is subsequent to the transformation of RecA protein into protease that cleaves LexA protein,

induced synthesis of RecA protein is itself an SOS function. The formation of RecA protease required for LexA repressor cleavage is the primary reaction that triggers the SOS pathway. It is dependent on the formation of a four-component structure , at least, that comprises single-stranded DNA, dATP, LexA repressor and RecA molecules (Phizicky and Roberts 1981; Little et al 1981); other proteins may also play a certain role such as the SSB protein (McEntee et al. 1980; Weinstock et al. 1981; Vales et al. 1980; Baluch et al. 1980). Induced synthesis of RecA protein is secondary to the activation of RecA protein.

Prophage λ repressor is hardly cleaved in vivo by RecA430 protein, and in vitro the rate of cleavage is 2% of that produced by wild type RecA protein (Roberts and Roberts 1981). If a similar four-component structure is involved in the cleavage of repressors of lambdoid phages (Craig and Roberts 1980), what is the deficiency brought about by RecA430 protein that makes the four-component structure not functional for cleavage of λ repressor?

Binding of RecA protein to DNA is a prerequisite for genetic recombination (Das Gupta et al. 1980; Radding et al. 1980; West et al. 1980). In contrast to what happens in recA13 mutants, genetic recombination appears almost normal in recA430 mutants as observed in Hfr x F^- crosses and by plating of λ*gam*$^-$ *red*$^-$ phages (Morand et al, 1977a). As opposed to recA13 mutants, no DNA degradation is observed in recA430 mutants in the absence of DNA damage, RecA430 protein can still control exonuclease V (Wright et al. 1971). We deduce from these facts that binding of RecA430 protein to DNA remains rather satisfactory and its relative impairement is not sufficient to account for the phenotype of recA430 mutants.

We observed that the various repressors studied displayed a decreased

susceptibility to RecA430 protease in the order: 80> LexA> 21> λ ≥ 434. It is clear that efficient cleavage depends on two parameters (i) the proteolytic activity of the enzyme (RecA protease) (ii) the nature of the substrate (repressor). Accordingly, the differential efficacy of RecA430 protease on various repressors may be accounted for by (i) the alteration of RecA430 protein that may extend beyond the proteolytic domain and affect RecA protein binding to repressor monomers (ii) the equilibrium between cleavable (monomeric) and non-cleavable (dimeric) repressor forms that is specific for a given repressor and may vary among them.

In support of the last assumption is the observation that high cellular concentrations of λ repressor prevented its inactivation (Bailone et al 1977) as can be demonstrated by monitoring repressor cleavage (Moreau, to be published). Resistance to cleavage has been related to λ repressor oligomerization, the cleavable form of λ repressor being the monomer (Roberts et al 1980).

Cohen and Little (1981) have demonstrated that prophage λ*ind*^{s} produces a population of repressor molecules consisting mainly of monomers and thus have accounted for the fact that prophage λ*ind*^{s} is induced at lower doses than is wild-type λ and produces phage λ*ind*^{s} spontaneously at a higher level in a *recA*$^+$ lysogen. Interestingly enough, induction of prophage λ*ind*^{s} in a *recA430* host reaches an efficiency of 5% whereas spontaneous production of phage λ*ind*^{s} is as low as that of λ (Dutreix, Bailone and Devoret, to be published). Sensitization of the 80 repressor to cleavage by RecA430 protease might be due to the fact that the population of prophage 80 repressor molecules might be constituted of monomers.

The differential activity of RecA430 protease on diverse repressors may reflect what already happens under physiological conditions, wild type

RecA protease possesses a different activity on LexA protein and on lambdoid repressors. Variable rates of inactivation of repressors will endow a cell with a clockwork mechanism. Some repressors (LexA protein) will be fully cleaved before others (e.g. prophage λ), this will provide a timing for sequential reactions: prophage λ DNA, induced by UV light, will begin to replicate when there will be enough induced RecA protein around so that prophage lesions will be efficiently repaired before replication.

The differential activity of RecA430 protease on diverse repressors may provide also an example and an explanation for the phenomenon of split phenotypes (Witkin 1976).

For instance, when an 80 lysogen carried either a lexA1 or a recA430 mutation or both, prophage 80 was fully induced by the presence of about 10 times less lesions than in a wild-type strain. Yet, we observed that the capacity of the mutant to reproduce the phage was altered, the burst of progeny phage being reduced 10 times. While they allow the occurence of prophage induction, the lexA1 and recA430 mutations confer to the bacterial host a visible deficiency in DNA repair that can be related to the impairement of two SOS functions, inducible error-prone repair (Quillardet et al 1981) and post-replicative recombinational repair (Sedgwick 1975). We assume that the repair deficiency resulting from RecA430 protease activity might delay the closing of single-stranded gaps left open for too long during the course of excision-repair following UV irradiation. Such DNA structures (Howard-Flanders et al 1967; George and Devoret 1971; Sussman and Ben Zeev 1975; Baluch and Sussman 1978) may constitute a vise ensuring the formation of a four-component structure (single-stranded DNA, RecA protein, dATP, repressor) that promotes the transformation of RecA protein into protease.

The RecA430 alteration entails a modified protease efficiency that varies according to the nature of the repressor to be cleaved. Some repressors (80 repressor) are more susceptible than others (LexA protein) as substrates of a weak protease; such differential susceptibility determines a dissociation in the induction of various SOS functions.

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LEGENDS TO TABLES

Table 1: Bacterial strains

The genotypes of the recA and lexA genes, the presence of plasmids and prophages, the origin and source of strains are respectively indicated in columns 2, 3 and 4.

Table 2: Phages strains

Phages are designated by our laboratory code name. The markers relevant to this study are indicated in columns 2, 3 and 4. The origin or source is indicated in column 5.

Table 3: Spontaneous and induced production of phage

The experiment was done as in fig. 1. The bacteria were exposed to 2 J/m^2 except for $lexA^+$ $recA^+$ lysogens which were given 20 J/m^2. Plating was done on GT plates without ampicillin. The frequency of infective centers and the percentage of clear plaques in parentheses are indicated in columns 6 and 7.

TABLE I

BACTERIAL STRAINS

Name	Genotypes		Prophages or plasmids	Origin or Source
	lex	rec		
AB1157	+	+	-	Howard-Flanders and Boyce (1966)
AB2463	+	A13	-	Howard-Flanders and Boyce (1966)
AT713	+	+	-	Taylor via Bachmann
C600	+	+	-	Appleyard via Wollman
DM455	+	A99	-	Mount et al 1971
DM456	+	+	-	Mount et al 1971
GY105	+	+	-	K12S hcr$^-$; Harm
GY158	+	+	-	KMBL49; Devoret and Blanco 1970
GY1049	+	A13	λ	AB2463
GY1139	+	+	F'15	van de Putte
GY1163	+	A430	-	derived from GY158
GY2375	+	A13	BA1	AB2463
GY3233	+	A430	F'15	Blanco
GY3236	+	A430	-	GY3233 x GY3450; Blanco
GY3239	+	A430	-	GY3236 cured of F'15 by acridine orange; Blanco
GY3255	+	A430	λ	GY3239
GY3422	+	+	-	Morand et al, 1977a
GY3424	+	+	-	GY3422 made Spc^r

Table I 2

GY3431	+	+	-	Morand et al, 1977a
GY3442	+	+	-	"
GY3448	+	A430	-	"
GY3450	+	+	-	KMBL1348 made Str^r
GY4015	+	+	-	C600 made Amp^R
GY4830	+	+	F82	GY105
GY4896	+	A430	BA1	GY3239
GY4909	+	A430	λ	JM776
GY4910	+	A430	BA1	JM776
GY4911	+	A430	BA1	GY3448
GY4912	+	A99	BA1	DM455
GY4913	+	A99	λ	DM455
GY4925	+	+	-	GY4015 x P4X6B, selection of *tonA*$^+$ *Amp*^{R} recombinants
GY4927	+	+	λt5 BC3	T5
GY4930	+	+	BC3	AB1157
GY4931	+	A430	BC3	JM776
GY4936	+	A430	FF1	JM776
GY4938	+	+	λt5 BC3	GY4927 made Mal^-
GY4939	+	+	BA1	AB1157
GY4941	+	A430	AB2	JM776
GY4942	+	A430	AC3	JM776
GY4943	+	+	F^+ λt5 BC3	GY4938 made F^+
GY4944	+	A430	BC3	GY3448
GY4945	A1	+	BA1	IC127
GY4946	A1	A430	BA1	IC128
GY4947	A1	+	λ	IC127

Table I 3

GY4948	A1	A430	λ	IC128
GY5222	+	A430	F74	GY3448
IC127	A1	+	-	Blanco
IC128	A1	A430	-	Blanco
JM776	+	A430	-	AB1157; Castellazzi
KMBL1348	+	+	-	Rörsch
P4X6B	+	+	-	Brenner via Signer
T5	+	+	λt5	Eisen
W1485	+	+	F+	Lederberg via Borek, alias GY2141

TABLE II

PHAGE STRAINS

Code name	Usual designation	Relevant markers			Origin
		imm	att	h	
A2	λ	λcI$^+$	λ	λ	Kaiser and Jacob
C21	λcI-26	λcI-26	λ	λ	Meselson
C40	λinm-203	λcI$^+$	λ	λ	Mount (1976)
E50	λ146	λv1v2v3	λ	λ	Bailone and Devoret
F74	λL26	λcI$^+$	434	λ	Kaiser and Jacob
F75	λL26c	λcI$^-$	434	λ	derived from F74
F81	F81	λcI$^-$	Δ9	80	Weisberg
F82	λh80	λcI$^+$	80	80	hybrid λ x 80
AA1	434J	434cI$^+$	434	434	Jacob
AA2	434c$^-$	434cI$^-$	434	434	derived from AA1
AB2	434T hy	434cI$^+$	λ	λ	Tomizawa
AC3	434cIind$^-$	434cIind-102	λ	λ	derived from AB2
BA1	80	80cI$^+$	80	80	Matsushiro
BC3	80cIind$^-$	80cIind-3	80	80	derived from BG9
BD1	80c	80cI-46	80	80	derived from BA1
BE1	80vir	80vir	80	80	Court
BG9	-	80cIind-3	supF	80	Brent
FF1	21hy-1	21cI$^+$	λ	λ	Kaiser
FF2	21hy-4	21cI$^-$	λ	λ	Kaiser

TABLE III

SPONTANEOUS AND INDUCED PRODUCTION OF PHAGE

Strain number	Background strain	Genotypes lexA	Genotypes recA	Prophage	Infective centers spontaneous	Infective centers induced
GY4939	AB1157	+	+	80	4×10^{-3}	1.0
GY4930	AB1157	+	+	BC3	7×10^{-6}(80)	4×10^{-4}(70)
GY4909	JM776	+	430	λ	7×10^{-6}(40)	3×10^{-6}
GY4910	JM776	+	430	80	4×10^{-2}(<1)	1.0
GY4931	JM776	+	430	BC3	10^{-6}(50)	3×10^{-5}(20)
GY3255	GY3239	+	430	λ	5×10^{-7}(75)	5×10^{-7}
GY4896	GY3239	+	430	80	10^{-2}(<1)	0.4
GY5222	GY3448	+	430	λL26	5×10^{-7}	2×10^{-6}
GY4911	GY3448	+	430	80	8×10^{-4}(<1)	0.15
GY4936	JM776	+	430	21hy-1	10^{-6}(<1)	5×10^{-4}(<1)
GY4941	JM776	+	430	λ434T	3×10^{-7}(90)	3×10^{-7}(100)
GY4942	JM776	+	430	AC3	3×10^{-7}(100)	3×10^{-7}(100)
GY4947	IC127	+	430	λ	1×10^{-3}(<1)	0.5
GY4945	IC127	+	430	80	5×10^{-2}	0.9
GY4948	IC128	1	430	λ	5×10^{-7}(100)	5×10^{-7}
GY4948	IC128	1	430	80	8×10^{-3}(50)	0.8(20)
GY1049	AB1157	+	13	λ	8×10^{-7}	6×10^{-7}
GY2375	AB1157	+	13	80	2×10^{-5}	2×10^{-5}
GY4913	AB1157	+	99	λ	10^{-6}	7×10^{-7}
GY4912	AB1157	+	99	80	2×10^{-5}	3×10^{-5}

LEGENDS TO FIGURES

Fig. 1. **One-step growth curve of a UV-induced recA430 (80) lysogen**

An overnight culture of GY4910:recA430 or of GY4939:recA$^+$ (80) lysogen grown in LBTU medium was diluted 1:100 into fresh LBTU medium and incubated with aeration up to OD_{650} = 0.35 corresponding to 2 to $3x10^8$ viable cells per ml.

The bacterial suspensions were centrifuged at 4°, the pellets resuspended in Tris 80, and GY4910: recA430(80) and GY4939:recA$^+$(80) exposed to 4 J/m^2 or 40 J/m^2 of UV light or not. The samples were diluted 1: 100 into prewarmed LBTU medium and incubated. As a function of time, 0.1 ml samples were removed, diluted and plated on GT-amp with GY4925. The infective centers were counted after overnight incubation.

Fig. 2. **Survival and induction curves of recA430 lysogens**

An overnight culture of λ or 80 lysogen grown in LBTU medium was diluted 1:100 into fresh LBTU medium and incubated with aeration up to OD_{650} = 0.35 corresponding to 2 to $3x10^8$ viable cells per ml.

The bacterial suspensions were centrifuged at 4°, the pellets resuspended in cold $MgSO_4$ 0.01 M for λ lysogens or Tris 80 for 80 lysogens.

Diluted samples were exposed to UV light or not and stored on ice before being plated on LA plates to measure surviving colonies. Infective centers were plated on GT-amp plates with 3 ml of soft agar containing 0.25 ml of an overnight culture of GY4925 indicator bacteria. Plates were counted after overnight incubation.

Solid symbols represent infective centers and open symbols colonies.

In panel A are the curves of derivatives of JM776: GY4910:recA430 (80) (Δ); GY4909:recA430 (λ)(○); GY4931:recA430 (80ind-3) (□); in panel B are the curves of derivatives of KMBL1348: GY4896:recA430 (80) (Δ); GY3255:recA430 (λ) (○); in panel C are the curves of derivatives of AT713: GY4911:recA430 (80) (Δ); GY5222:recA430 (F74) (○).

Fig. 3. **Induction pattern of BC3:80ind-3 in AB1157**

Strains GY4939 (80) (triangles) and GY4930 (BC3) (squares) were treated as in figure 2.

Fig. 4. **Induction patterns of 80 and λ lysogens carrying lexA1 or lexA1recA430**

Strains GY4945:lexA1 (80) (triangles), GY4946:lexA1 recA430 (80) (squares), GY4947:lexA1 (λ) (circles) and GY4948:lexA1 recA430 (λ) (inverted triangle) were treated as in figure 2.

Fig. 5. **Induction patterns of various lambdoid prophages**

The strains were treated as in figure 2. Solid symbols represent infective centers and open symbols colonies. In the figure are the curves of derivatives of JM776: GY4936:recA430 (FF1) (○); GY4941:recA430 (AB2)(Δ); GY4942:recA430 (AC3) (▽).

Fig. 6. **Rate of RecA protein synthesis in UV-irradiated bacteria**

The four bacterial cultures AB1157, IC128, JM776, IC127 were grown at 37° in enriched YM9 medium (Moreau et al 1980) plus LB 2% and 20 µg/ml of cystein and 30 µg/ml of methionine (medium A) up to an A_{650}= 0.4, then they

were diluted 3-fold in enriched YM9 medium plus LB 2% containing 20 µg/ml of cystein and 10 µg/ml of methionine (medium B). The diluted cultures were incubated up to an A=0.3 - 0.4.

At time 0 (before UV-irradiation), a sample of each incubated culture was taken and labeled with ^{35}S-methionine (30 µC/ml, 1000 C/mmol) for 10 min at 37°. Labelling was stopped by adding to the cell suspensions 200 µl of cold YM9 containing 10 mM NaN_3 and 500 µg/ml methionine.

Exposure to UV-light was done as follows. The cell suspensions were centrifuged, the pellets resuspended in 10 mM $MgSO_4$ before exposure to UV-light. The irradiated cells were then resuspended in pre-warmed medium B incubated at 37° with agitation for 20 min and labeled as described above.

Then the cell suspensions were processed for precipitation of RecA protein with an anti-serum; the precipitated proteins were analyzed by SDS-PAGE on a 10% acrylamide gel as described previously (Moreau et al 1980a) with some slight modifications to be detailed elsewhere (Moreau et al 1981). On the autoradiograph the position of the RecA protein is indicated by an arrow.

Fig. 7. **Rocket immunoelectrophoresis of RecA protein**

The eight bacterial cultures AB1157, IC128, JM776, IC127 and their 80 lysogenic derivatives GY4939, GY4946, GY4909, GY4945 were grown at 37° in enriched YM9 medium (Moreau et al 1980) plus LB 2% and 20 µg/ml of cystein and 30 µg/ml of methionine up to an A_{650}= 0.3. UV-irradiation was performed as indicated in Fig. 6. After irradiation, the cell suspension was further incubated for 30 min at 37°.

At time 0 (before UV-irradiation), 25 ml samples were removed, mixed with 5 ml of cold YM9 containing 10 mM NaN_3 and processed for immu-

noelectrophophoresis (IEP). The exposure dose to UV-light is indicated on the graph. The crude extracts were prepared as described by Roberts and Roberts (1975).

Then, 10 µl aliquots of the crude extracts were put into the wells of a horizontal 1% agarose gel containing 5% anti-RecA protein serum. Electrophoresis was done at 80 V for 18 h. The immunoprecipitates were stained with Amido-Black. Peak areas are proportional to the amount of RecA protein (Laurell 1966). A standard of pure RecA protein is on the far right.

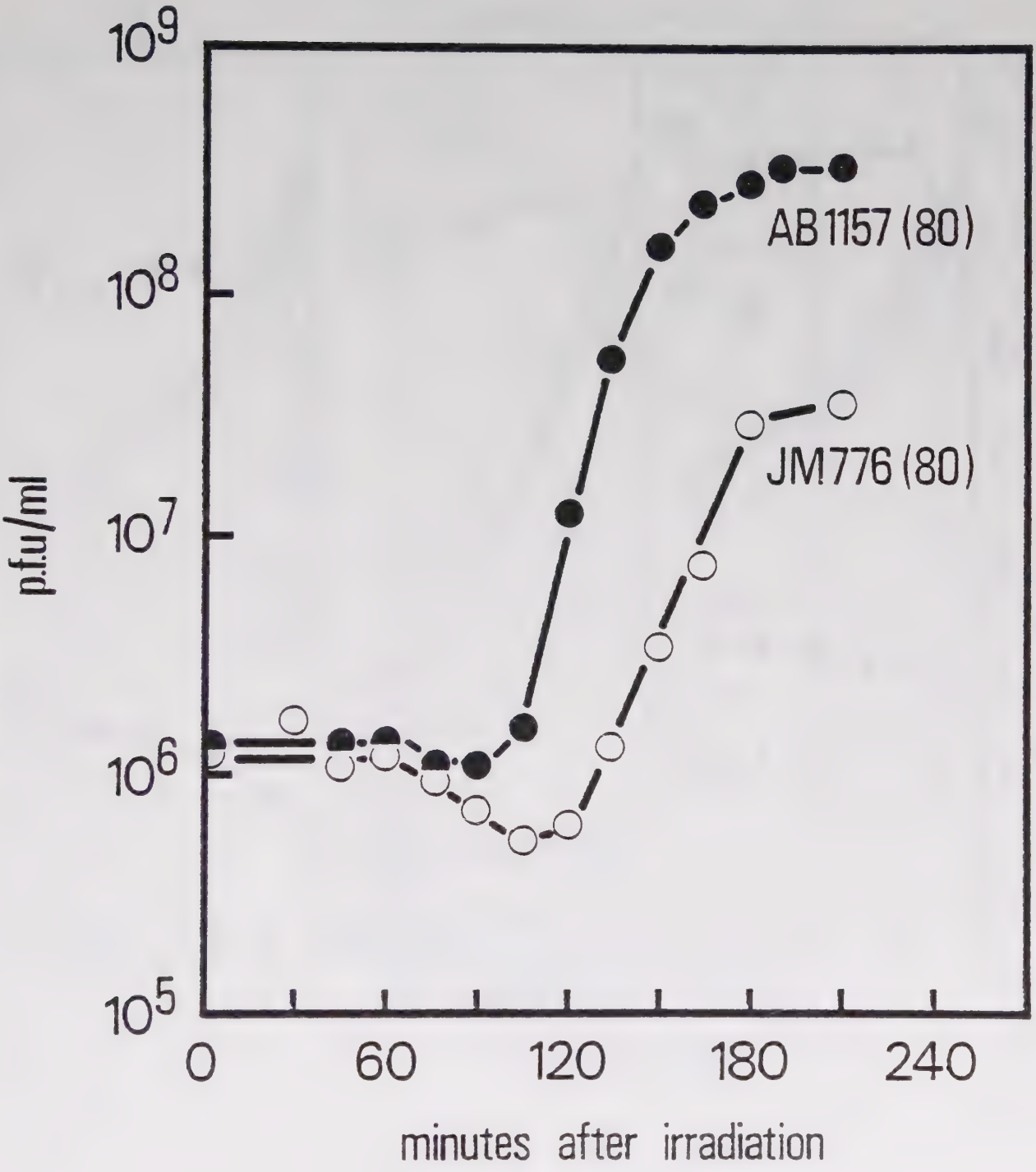

10^9
10^8
10^7
10^6
10^5
p.f.u/ml
AB 1157 (80)
JM 776 (80)
0
60
120
180
240
minutes after irradiation

FIG. 1

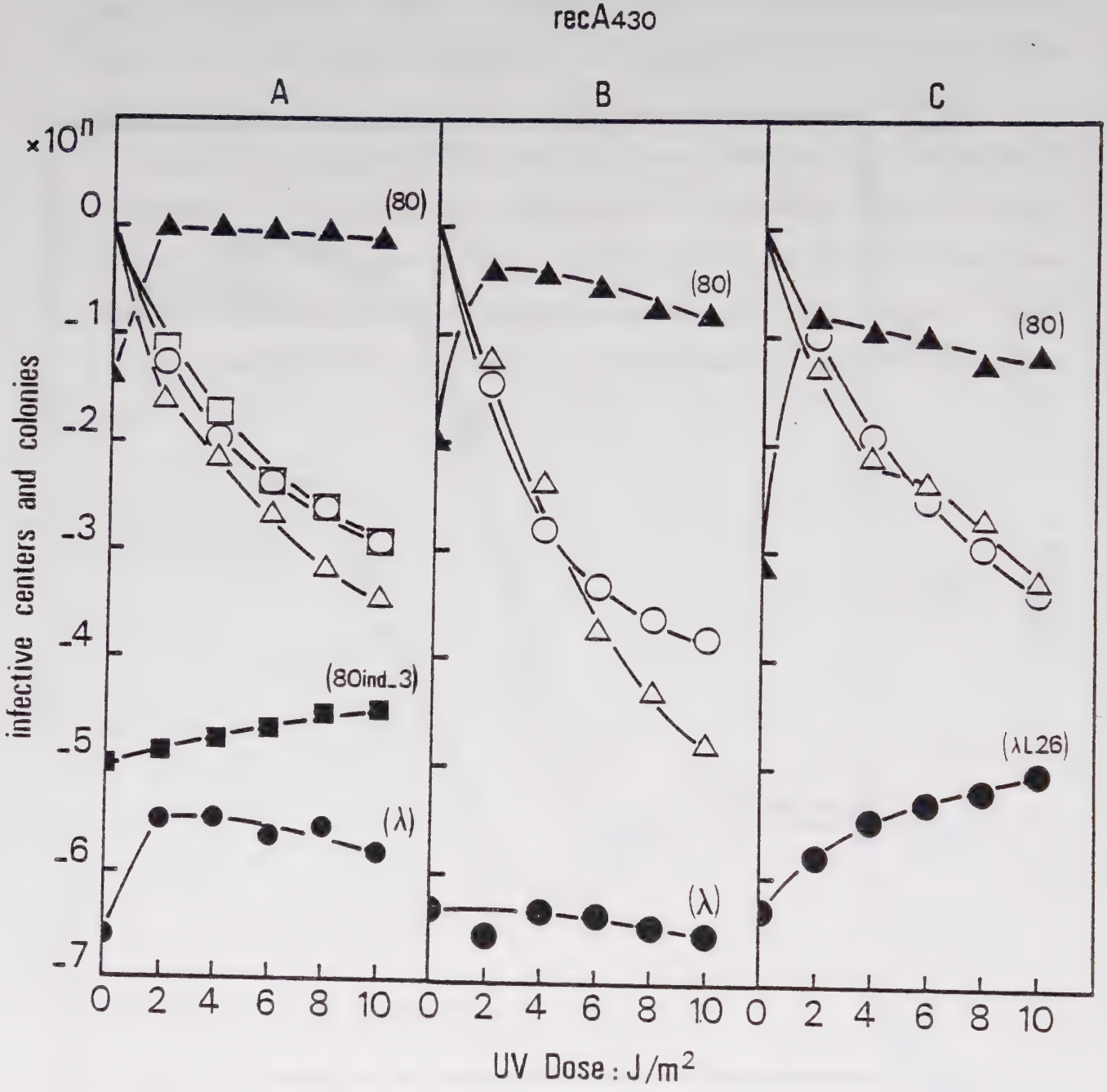

recA430
A
B
C
×10^n
0
-1
-2
-3
-4
-5
-6
-7
(80)
(80ind-3)
(λ)
(λL26)
infective centers and colonies
0 2 4 6 8 10
UV Dose : J/m²

FIG. 2

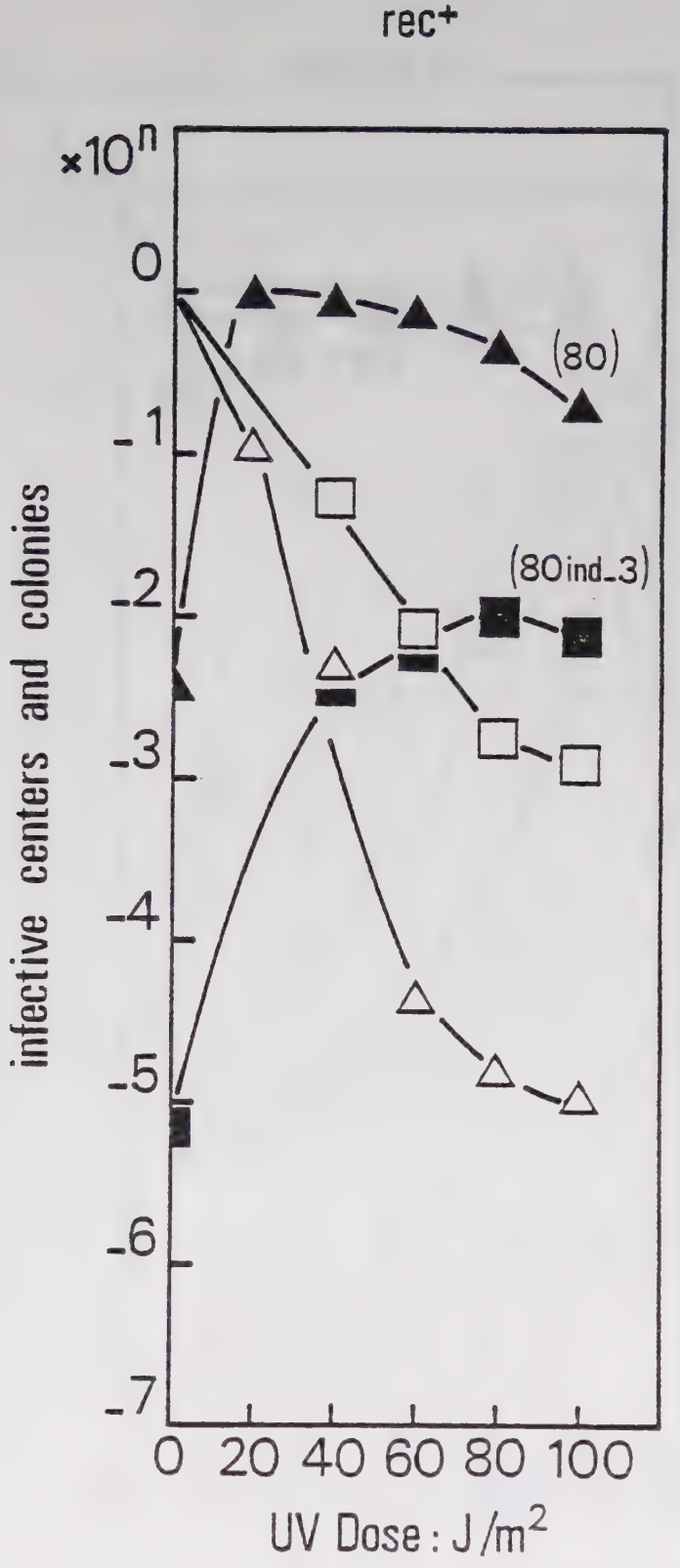

rec+
×10^n
0
-1
-2
-3
-4
-5
-6
-7
(80)
(80ind-3)
infective centers and colonies
0 20 40 60 80 100
UV Dose : J/m²

FIG.3

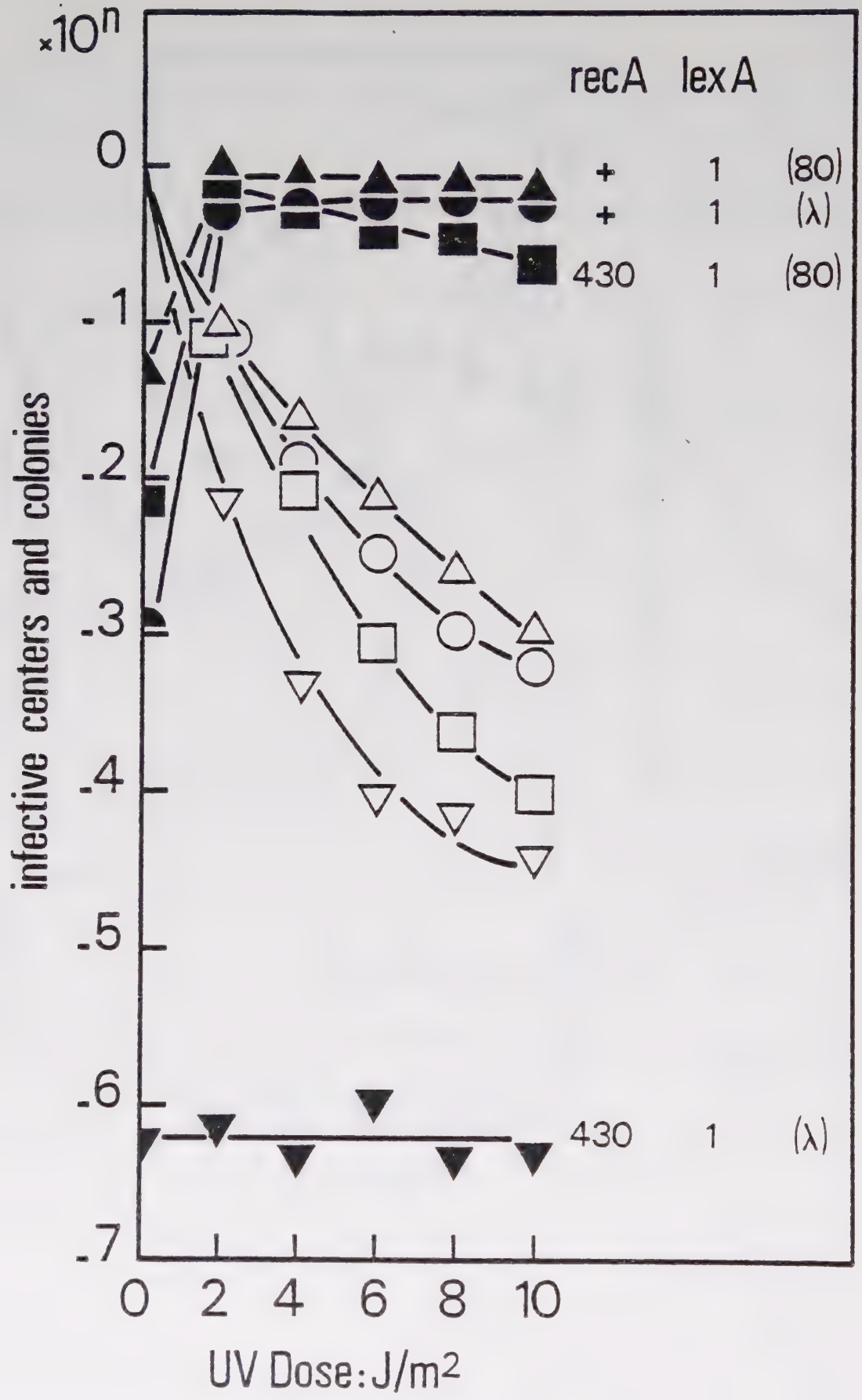

×10^n
recA lexA
0
-1
-2
-3
-4
-5
-6
-7
+ 1 (80)
+ 1 (λ)
430 1 (80)
430 1 (λ)
infective centers and colonies
0 2 4 6 8 10
UV Dose: J/m²

FIG.4

recA430

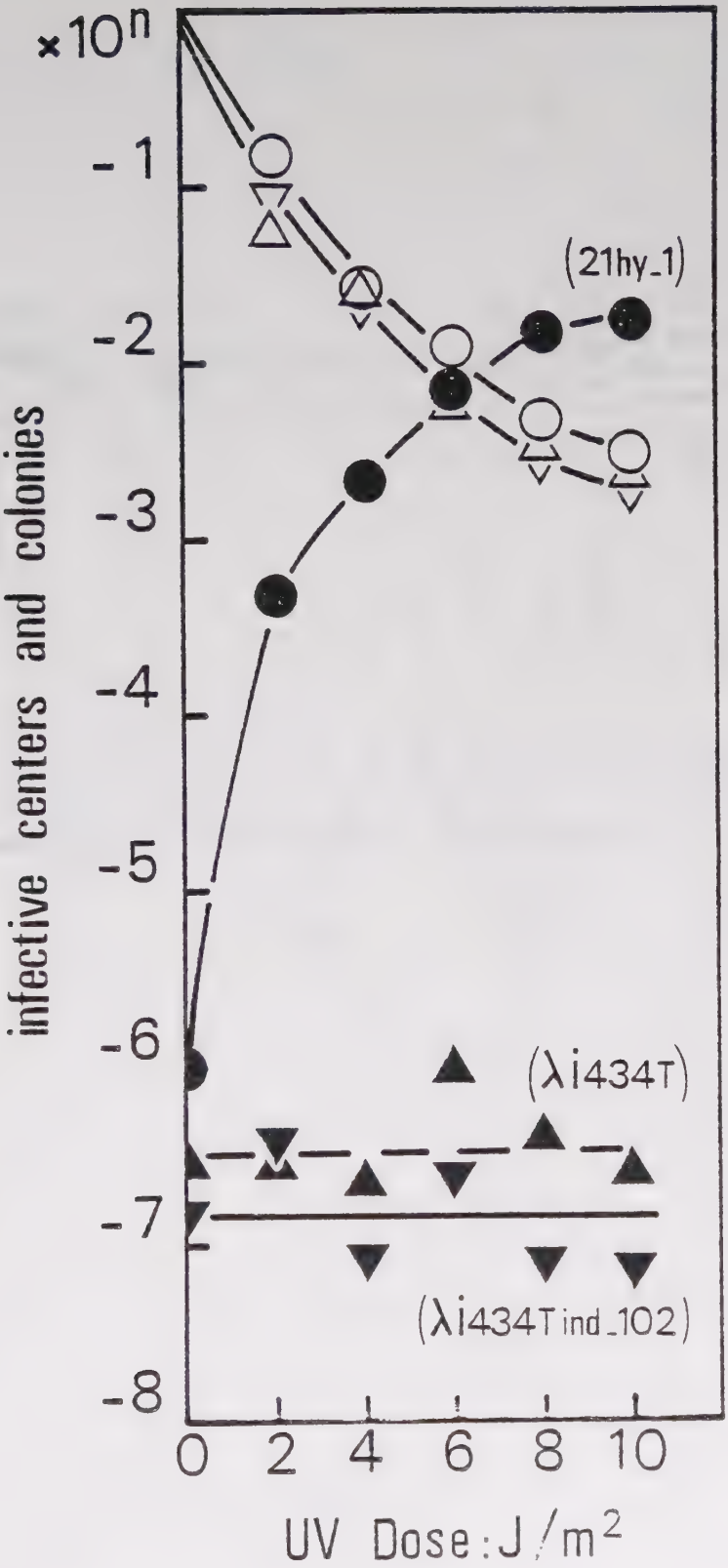
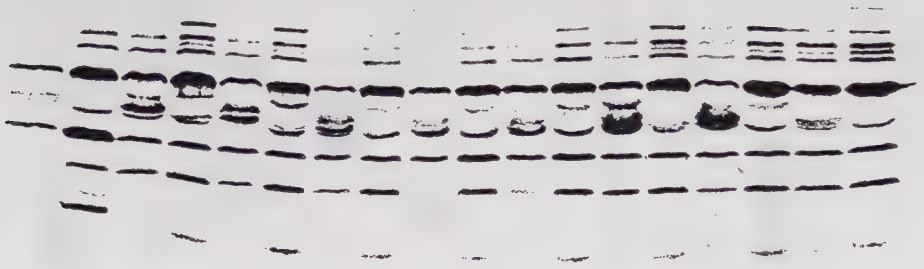


FIG.5



J/m²	0	60	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	4
prophage	-		-		80		λ		80		λ		-		80		λ	
lexA	+		1						+				1					
recA	+		430						430				+					

FIG.6

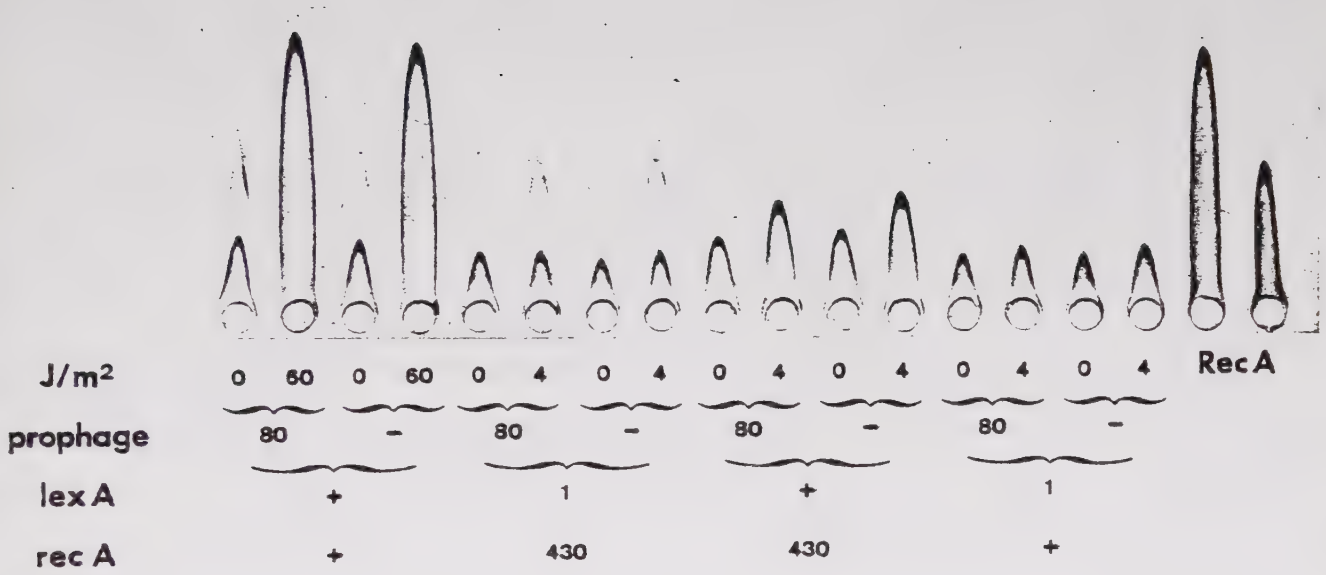

J/m²
0 60 0 60 0 4 0 4 0 4 0 4 0 4 0 4
RecA
prophage
80 – 80 – 80 – 80 –
lex A
+ 1 + 1
rec A
+ 430 430 +

FIG.7

Cleavage of λ repressor and synthesis of RecA protein induced by transferred UV-damaged F sex factor

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SUMMARY

Transfer of a UV-damaged F sex factor to a recipient λ lysogen induces prophage λ development. Under these conditions we showed that RecA protein synthesis was induced and λ repressor cleaved as observed upon direct induction, that is, when the recipient λ lysogen is directly exposed to UV-light. The efficiency of induction of RecA protein synthesis in recipient bacteria which had received an irradiated F-lac factor was about 80% of that measured upon direct induction. We observed the simultaneous disappearance of λ repressor and a slight production of cleavage fragments; quantitation by densitometric scanning of the autoradiogram indicated that 55% of λ repressor was then cleaved when corrected for the efficiency of transfer. Transfer of UV-damaged Hfr DNA failed to induce RecA protein synthesis. A λ phage vector carrying oriF, the cloned origin of replication of the F plasmid, after exposure to UV-light and infection of a recipient lysogen induced RecA protein synthesis and a moderate but significant cleavage of λ repressor. Indirect induction by UV-damaged F sex factor or phage λoriF results in biochemical cellular reactions similar to those observed upon direct induction. Yet LexA repressor that controls negatively RecA protein synthesis appeared more susceptible to cleavage than λ repressor.

INTRODUCTION

In Escherichia coli, treatments that damage DNA or inhibit DNA synthesis lead to the expression of a set of coordinated cellular functions (SOS functions) that aid bacteria and their phages to survive. These functions include induced synthesis of RecA protein, inhibition of cell division, inducible error-prone repair, and prophage induction (see reviews by Witkin 1976 and by Devoret 1981). Recent evidence shows that induction of RecA protein synthesis and prophage λ development are caused by RecA protein that cleaves two repressors i) LexA protein, the repressor of the recA gene (Little et al. 1980 ; Little et al. 1981) and ii) the λcI protein (λ repressor) (Roberts et al. 1978 ; Roberts and Roberts 1981).

It has been surmised that induction of prophage λ requires induction of RecA protein synthesis (McEntee 1977 ; Gudas and Mount 1977 ; Roberts et al. 1978 ; Mount 1979 ; Satta et al. 1979 ; Craig and Roberts 1980). This assumption derived mostly from the fact that proteolytic cleavage of λ repressor in induced cells requires de novo protein synthesis (Shinagawa et al. 1977). However, we have shown that full cleavage of λ repressor occurs when induction of RecA protein synthesis is greatly reduced by rifampicin (Moreau et al. 1980a). In recA$^+$ lysogens, the basal cellular level of RecA protein can give rise to enough RecA protease molecules to induce prophage λ. Transformation of RecA protein into protease in response to chromosomal damage appears therefore as the critical event for the derepression of the recA gene and prophage λ.

In vitro, RecA protease requires the formation of a three-component complex RecA protein - dATP - single stranded DNA (Phizicky and Roberts 1981 ; Craig and Roberts 1981). Is the formation of this complex sufficient to cleave LexA and λ repressors in vivo ?

The mechanism of in vivo activation of RecA protein can be approached by the study of "indirect induction". In effect,prophage λ induction occurs when a λ lysogenic F^- recipient is mated with a ultraviolet (UV)-irradiated F^+ donor (Borek and Ryan 1958). Since the induced λ lysogen is not exposed to the inducing agent, this phenomenon has been called indirect induction. A variety of plasmids such as the ColI colicigenic factor or phage P1 cause indirect induction. Genetic experiments established that a UV-irradiated plasmid triggers prophage λ induction because it can initiate its replication into recipient cells (Devoret and George 1967 ; Rosner et al. 1968 ; Monk 1969 ; George and Devoret 1971).

By using irradiated small replicons, we sought to define the requirements for induction of prophage λ, to establish whether irradiated replicons trigger a pathway similar to that assumed to lead to the activation of RecA protein upon direct induction. Alternatively, it was assumed that indirect induction might also result from genetic exchanges between the damaged replicon and the host chromosome (Benbow et al. 1973) or from the trapping of λ repressor to damaged DNA (Sussman and BenZeev 1975 ; Sussman et al. 1978). To differentiate between these processes, we measured whether RecA protein synthesis was induced and λ repressor cleaved upon indirect prophage λ induction.

MATERIALS AND METHODS

Bacterial and phage strains.

Escherichia coli K12 derivatives used are listed in table 1. Strain GY4915 was cured of its F sex factor with acridine orange. Curring was assessed by resistance to "male" phage MS2 (Miller 1972). Mal^- Lam^R derivatives of lysogenic bacteria were obtained as in George et al. (1974). Phage λoriF (λpSC138) carries the EcoR1 f5 fragment of F sex factor that contains the F replication origin (Couturier et al. 1979).

Media

Medium A was Na_2HPO_4 : 41 mM, KH_2PO_4 : 22 mM, NH_4Cl : 18.5 mM, NaCl : 85.5 mM, $MgCl_2$: 0.5 mM, $CaCl_2$: 0.1 mM containing thiamine: 1 µg/ml , glucose: 0.2 % and 14 L-amino acids except the sulfur containing amino-acids at 50 µg/ml each. Medium A supplemented with LB broth : 2 %, thymine : 8 µg/ml, uracile : 8 µg/ml and $MgSO_4$: 200 µM gave B medium. Different from B medium, media C and D contained respectively 50 µM and 10 mM $MgSO_4$. Enriched YM9 medium was as described elsewhere (Moreau et al. 1980b).The division time of bacteria grown in the media described above was 40 min (rec^+ bacteria) or 70 min (uvr^- rec^- bacteria) at 37°C. The turbidity of cultures was monitored with a Lange photocolorimeter : a turbidity value of 0.15 corresponded to a cell concentration of $2x10^8$ bacteria per ml.

Other media : LB broth, YM9, GT-amp medium plate, and soft agar were as described previously (Moreau et al. 1976 ; Moreau and Devoret 1977, Moreau et al. 1980b). EMBlac medium

plates (Devoret et al. 1981) containing 100 µg/ml of streptomycine were used to measure the efficiency of conjugation.

UV-irradiation

Bacteria or phage suspensions were irradiated with a 15 W germicidal lamp. UV irradiation was performed at a dose of 0.1 or 1 J/m^2 per sec as measured with a Latarjet dosimeter. UV-irradiation was carried out under yellow light and all incubations were in the dark.

Measurement of RecA protein synthesis.

1. Slab gel electrophoresis. The previously described procedure (Moreau et al. 1980b) was modified to prevent degradation of RecA protein. Labeled cells were pelleted and resuspended in 25 µl of 10 mM Tris.HCl at pH 7.9 containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM KCl, and 0.6 mg/ml lysozyme (Worthington). The cells were subjected to six cycles of freezing and thawing to produce crude extracts. To the extracts were added 140 µl of 50 mM Tris.HCl at pH 7.5 containing 2 M KCl, 2 % Triton X-100 (Koch Light) and 0.66 % polymin P (Miles), and the mixtures were incubated at 0°C for 30 min with gentle agitation. Debris and DNA were pelleted by centrifugation in an Eppendorf minicentrifuge for 15 min. The supernatants (140 µl) were mixed in small glass tubes with 25 µl of Tris.HCl at pH 7.5 containing 2 M KCl, 2 % Triton X-100, and 5 % anti-RecA serum (Moreau et al. 1980b). After 1 h incubation at 0°C with vigorous agitation, 30 µl of protein A-Sepharose CL-4B (Pharmacia) were added and incubation was continued with agitation for at least 1 h. The suspensions were

then poured into small columns and washed with 2 ml of cold 0.1 M Tris.HCl at pH 6.8 containing 0.5 M LiCl, and 1 % 2-mercaptoethanol. The immune complexes were then eluted with 40 µl of final sample buffer (125 mM Tris.HCl at pH 6.8 containing 2 % sodium dodecyl sulfate, 10 % glycerol, 10 % 2-mercaptoethanol, and 0.0015 % bromophenol blue) at 45°C, heated for 1 min at 100°C and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Favre 1973). Electrophoresis was done at a constant current intensity (30 mA) for about 4 h. Proteins were fixed in the gel for 1 h with 50 % methanol- 10 % acetic acid. The gels were finally dried and placed in contact with X-ray films (Kodak) for about 4 days. The optical density of the RecA band was measured on the autoradiograms with a Transidyne RFT scanning densitometer coupled to a computing integrator.

2. "Rocket"immunoelectrophoresis. The crude extracts were prepared as described by Roberts and Roberts (1975). The cells were lysed by the freeze-thaw lysozyme technique. After addition of 50 µl of 10 mM Tris.HCl at pH 7.9 containing 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM KCl, 5 % glycerol, 20 µg/ml deoxyribonuclease I (Worthington), and 10 µg/ml ribonuclease A (Worthington), the extracts were incubated at 20°C for 30 min. Debris was removed by centrifugation. Then 10 µl aliquots of the crude extracts were put into wells of a horizontal 1 % agarose gel containing 5 % anti-RecA serum (Laurell 1966). Electrophoresis was done at 80 V for 18 h. The gels were washed, dried and the immunoprecipitates were stained with Amido-Black. Under our experimental conditions, the peak heights were proportional to the

amount of RecA protein. When the heights of the peaks were between 30 and 60 mm , exact quantitation was carried out by measuring the areas enclosed by the precipitates.

Immunological titration of λ repressor was done as previously described (Moreau et al. 1980b).

Other methods.

The [^{35}S] radioactivity incorporated into proteins was determined by pipetting 5 µl of crude extracts into 1 ml of 10 % (wt/vol) trichloroacetic acid. After standing on ice for 1 h, the precipitates were collected on GF/C filters (Whatman) and washed three times with 5 ml of 10 % trichloroacetic acid and one time with 95 % ethanol. The filters were dried, put in Aquasol (N.E.N.) and then radioactivity counted in a Intertechnique liquid scintillation counter. Protein concentration in crude extracts was measured by the Bio-Rad protein assay.

RESULTS

1. Time course of indirect induction

In order to measure RecA protein synthesis and proteolytic cleavage of λ repressor in the recipient of a mating mixture, we had to grow bacteria in synthetic medium to label cellular proteins with ^{35}S. Although it had been reported that the efficiency of indirect induction was variable when bacteria were grown in minimal medium (Devoret and George 1967), using enriched YM9 medium and its derivatives, we obtained reproducible results. Induction of RecA protein synthesis in male bacteria was prevented by using donors carrying the uvrB and recA13 or ΔrecA mutations. It is known that in donor bacteria (i) the absence of excision repair increases the efficiency of indirect induction and ii) a recA$^-$ mutation does not affect indirect induction of prophage λ (George and Devoret 1971).

Wild type λ lysogenic bacteria W1177 were readily induced upon mating with GY3959: uvrB recA13/F'lac$^+$pro$^+$ bacteria irradiated at 80 J/m^2(Fig. 1). The optimal UV-dose and the number of free phage after 120 min of incubation were similar to that measured when bacteria were grown in rich LB medium (Devoret and George 1967 ; George and Devoret 1971). However, in contrast to what had been described when bacteria were grown in rich medium, we observed that under our experimental conditions the optimal ratio of F' to F$^-$ bacteria was from 1.5 to 8. After 30 min of mating, 60 % of the recipient bacteria CSH7 : lacY1 were Lac$^+$ indicating a high frequency of F-lac transfer. Achtman (1975) estimated that at most 50 % of the females can receive male DNA when the ratio male/female is high. The pro-

duction of free phage was also maximal upon mating with irradiated F-lac factor (data not shown). The phage yield per infective center estimated to be about 150 (Fig. 1) was comparable to that measured after direct ultraviolet induction of lysogenic bacteria.

The fact that lysis of induced lysogens occurred upon indirect induction about 15 min later than upon direct induction may be accounted for by the observation that a delay of about 12 min after mating is required for promoting indirect induction with a large F' factor (Rosner et al. 1968).

Our results confirmed that indirect induction of prophage λ occurs rapidly after the introduction of a UV-damaged F sex factor into λ lysogens. However, the efficiency of indirect induction, i.e., the fraction of lysogenic bacteria induced, was relatively low : only 20 % of the lysogens which had received a damaged F sex factor were induced as compared to the efficiency of transmission of the F-lac factor,which is not affected by irradiation (Monk 1969, George and Devoret 1971),

2. RecA protein synthesis was induced in a recipient upon mating with a UV-irradiated F' donor.

Induction of RecA protein synthesis is measured by pulse-labeling of cellular proteins and by one- (Gudas and Pardee 1976) or two-dimensional polyacrylamide gel electrophoresis (McEntee 1977). The two procedures do not permit, however, to measure RecA protein precisely enough since : 1) proteins other than RecA have the same isoelectric point and size (Baluch

et al. 1980) ; 2) RecA protein is subject to a limited proteolysis when detached from DNA (McEntee 1977 ; Baluch et al. 1980 ; Moreau et al. 1980b). We circumvented these two difficulties by precipitating RecA protein with a specific antiserum and preparing crude extracts with a procedure described by Roberts and Roberts (1975) that we modified as follows. Immediately after cell lysis by the freeze-thaw lysozyme technique, RecA protein was detached from the bulk of DNA by addition of detergent, salt, and polymin P, the incubation step with nucleases being omitted (Fig.2).

The difference between the kinetics of cellular protein synthesis observed in the heterosexual (Fig. 3B) and homosexual (Fig. 3C) mating mixtures was expected. It is clear that in the mating mixture 3B, the donor synthesized only plasmid-coded proteins. Sancar et al. (1979) observed that synthesis of chromosome-coded proteins decreased rapidly in highly UV-irradiated bacteria carrying uvr$^-$ and recA$^-$ mutations whereas, if these cells contained a plasmid, the plasmid-coded proteins continued to be synthesized. Since in the mating mixtures the ratio of UV-irradiated F'donors to recipient bacteria was about 8, the percentage of plasmid-coded proteins in a uvr$^-$ recA$^-$ donor corresponded to about 5 % of the chromosome-coded proteins in the recipient. This result is in good agreement with the size of the F' factor relative to the chromosome.

The rate of synthesis of RecA protein increased markedly 15 min after mating recipient bacteria with UV-irradiated donor uvr$^-$ recA$^-$ bacteria (Fig. 3B). It was 3-fold higher in the heterosexual (Fig. 3B) than in the control homosexual mating mixture (Fig. 3C) after 45 min. A similar kinetics

of induction was observed when the recipient tester bacteria were induced by direct UV-irradiation (Fig. 3A) : the rate of RecA protein synthesis increased 8-fold whereas the rate of total protein synthesis decreased 3-fold as compared to the control mating mixture (Fig. 3C).

Two sets of experiments confirmed this result.

Firstly, the amount of RecA protein synthesized between 30 and 60 min after mixing female with irradiated "donor" bacteria was 7.5 higher in the heterosexual (Fig. 4, lane c) than in control homosexual mixture. Note that in this experiment, for 30 min the bacteria were gently agitated for efficient mating and then incubated with vigorous agitation to allow rapid metabolism.

Secondly, Fig. 5 shows the results of a quantitative estimation of RecA protein by electrophoresis in agarose gel containing anti-RecA serum (rocket immunoelectrophoresis) (Laurell 1966).

Rocket immunoelectrophoresis must be carried out at a low ionic strength, consequently we could not use the new procedure for preparing crude extracts as for SDS-PAGE. Note that when purified RecA protein or cellular extracts were tested the aspect of the peaks was unchanged. When RecA protein was not dissociated with sodium dodecyl sulfate (SDS) and was likely in a tetrameric form (McEntee et al. 1976), there was no degradation of RecA protein as observed on SDS-polyacrylamide gel (Moreau et al. 1980b). If degradation products had been produced, several lines of precipitates would have appeared.

Rocket immunoelectrophoresis (IEP) provides a good quantitative estimate of RecA protein in crude extracts.

Fig.5 shows that after 80 min of incubation of mating mixtures, the cellular level of RecA protein in recipient F^- rec^+ bacteria when mated with irradiated F' $\Delta recA$ bacteria was 1.7-fold higher (well g) than when mated with non-irradiated F'$\Delta recA$ bacteria (well f) or irradiated $F^-\Delta recA$ bacteria (well e). Since upon indirect induction, the cellular level of RecA protein was at least 50% of that measured upon direct induction (compare wells g and d), this result confirms that shown on Fig.3. We estimated that upon indirect induction the efficiency of induced RecA protein synthesis was about 80% of that measured upon direct induction since about 60% of the recipient bacteria had received an irradiated F-lac factor.

The accumulation of RecA protein found in recipient bacteria when mixed with an excess of "donor" bacteria (Fig.5) was minimal because of the high cell density used, which prevented efficient cell aeration and consequently rapid metabolism (data not shown). Upon optimal incubation, the increase in the cellular level of RecA protein was linear for at least 60 min (Fig. 6) after direct UV-irradiation of the recipients at relatively high doses (Fig. 7) ; the level of RecA protein was then about 12-fold higher than in non-irradiated bacteria.

When recipient bacteria were mated for 80 or 120 min with Hfr recA1 donors irradiated at 80 J/m^2, no increase in the cellular level of RecA protein was detected by rocket-IEP (data not shown). This result confirms the failure of an Hfr

KL16 donor to mediate indirect induction.

3. Indirect induction of λ repressor cleavage

Indirect induction of RecA protein synthesis provided indirect evidence of LexA protein cleavage by RecA protein. LexA protein might have been trapped on damaged DNA: when LexA protein is modified as a consequence of a spr⁻ mutation, the synthesis of RecA protein is derepressed but the cleavage of λ repressor does not occur (McEntee 1977). In the absence of DNA damage, a high cellular level of RecA protein fails to induce prophage λ development. Cleavage of λ repressor demonstrates the formation of RecA protease.

That λ repressor is cleaved is demonstrated by the appearance of cleavage fragments called R' (Roberts and Roberts 1975). To facilitate the detection of these fragments, we used GY4816 : pKB202 bacteria, which overproduce 5-fold more λ repressor than λ monolysogens, such as GY3639. It is known that the rate of inactivation of λ repressor is similar in both strains (Bailone et al. 1979). Fig. 8 shows that characteristic R' fragments were produced when irradiated F' donors were mated for 80 min with GY4816 : pKB202 recipient bacteria. Note that upon direct induction of the same strain (Fig. 8A), λ repressor molecules were not all cleaved : after 80 min of incubation, the cellular level of λ repressor corresponded to that of the monolysogen GY3639 (Fig. 8E).

Upon indirect induction of GY3639 : (λ) (Fig. 8G), we could observe simultaneously the disappearance of λ repressor (R) and a slight production of cleavage fragments (R') ; quantitation by densitometric scanning of the autoradiogram indica-

ted that 35 % (or 55 % when corrected for the efficiency of transfer) of λ repressor was then cleaved.

In Fig. 8C and F, the radioactive label decreased equally in all peaks because in the mating mixtures unirradiated F' donors continued to grow and thus labeled proteins of "lysogenic" bacteria were somewhat diluted. The slight decrease of the R peak was artifactual since it was not correlated with the increase of R' in contrast to what was observed upon mating with a UV-irradiated donor (Fig. 8D and G).

4. UV-irradiated phage λoriF induced the synthesis of RecA protein.

In contrast to phage λ, a UV-irradiated vector phage λ bearing the origin of replication of F (λoriF) induces bacteria lysogenic for prophage ϕ80 upon infection (Devoret 1981). The similarity of effect of irradiated F factor and irradiated phage λoriF prompted us to test the ability of irradiated phage λoriF to induce RecA protein synthesis. Figs 9 and 10 show the dose-dependent induction of RecA protein synthesis in GY4816 : pKB202 bacteria infected with UV-irradiated phage λoriF. The optimal UV-dose needed for maximal indirect induction of RecA protein synthesis with phage λoriF was similar to that measured with the F sex factor.

The optimal multiplicity of infection (m.o.i.) was between 10 and 20 ; the efficiency of induction plateaued, in effect, it decreased by less than 20 % when the m.o.i. was up to 40 (data not shown). This indicates that the probability of induction increased when the cells were multiply infected

in contrast to what was found with phage P1 (Rosner et al. 1968). Whatever the m.o.i. used within a range from 5 to 40 neither non-irradiated phage λoriF nor the vector phage, irradiated or not, affected the cellular concentration of RecA protein upon infection of GY4816 : pKB202 bacteria (data not shown). It should be noted that upon infection of GY4816 bacteria overproducing λ repressor, irradiated phage λoriF did not replicate as a phage but as a plasmid (Couturier et al. 1979).

The efficiency of indirect induction of RecA protein synthesis was 1.5-fold higher with irradiated phage λoriF than with irradiated F-lac factor. This is likely due to a higher efficiency of transfer of damaged DNA by infection than by mating.

Surprisingly enough, the ability of irradiated phage λoriF to promote induction of RecA protein synthesis was not correlated with its ability to promote cleavage of λ repressor. Only a moderate cleavage of λ repressor was found. This activity was, however, inhibited when infection was performed in the presence of 100 µg/ml of chloramphenicol (data not shown). The apparent discrepancy between induction of RecA protein and poor cleavage of λ repressor may be accounted for by the assumption that only free λ repressor molecules in a monomeric form can be cleaved by RecA protease (Phizicky and Roberts 1980). Repressor molecules which bind rapidly in a dimeric form to the operator sites carried by infecting phage λoriF were likely resistant to cleavage.

DISCUSSION

In this paper we provide evidence that "direct" and "indirect" prophage λ induction result from the same molecular mechanism. Upon indirect induction, synthesis of RecA protein in the recipient lysogens was induced almost immediately upon mating with UV-damaged F' donors and λ repressor was cleaved. Introduction into a lysogen of a UV-damaged replicon activates RecA protein which, then, cleaves LexA protein (Little et al., 1980) and prophage λ repressor (Roberts et al., 1978). In contrast, Hfr bacteria that carry the F sex factor in an integrated state did not cause indirect induction of RecA protein synthesis.

Our results suggest that upon indirect induction cleavage of LexA protein occurred at a high efficiency because in almost each bacterium which had received a damaged F' factor RecA protein synthesis was fully induced. In contrast, cleavage of λ repressor was less efficient and only 20% of the recipient lysogens which had received a damaged F-lac factor were induced to produce free phage. In other words, induction of RecA protein synthesis appeared almost as an all-or-none process whereas cleavage of λ repressor appeared to stop before completion.

Our results indicate that upon indirect as well as direct induction (i) prophage λ is induced more slowly than RecA protein synthesis, and (ii) the "inducing signal" that activates RecA protein to cleave repressors is short-lived. In effect, upon direct induction, it is known that incomplete

cleavage of λ repressor without subsequent prophage induction may occur in a cell : prophage λ induction occurs in a lysogen only when 90% of the λ repressor molecules are inactivated (Bailone et al. 1979). On the other hand, synthesis of RecA protein is induced right after UV-irradiation whereas there is a 10 min lag period before one can detect λ repressor cleavage (Moreau et al. 1980a). Little et al.(1981) showed that in vitro cleavage of LexA protein proceeded more rapidly than cleavage of λ repressor. We have found that upon direct induction, RecA protein synthesis was fully induced at a UV-dose about 5-fold lower than that required for induction of prophage λ. Induction of prophage λ appears, therefore, to require a longer lenght of time and a higher UV-dose than induction of RecA protein synthesis. These results and the fact that induction of RecA protein synthesis was partially uncoupled from prophage λ induction upon indirect induction when damaged DNA is limited are in good agreement with the notion that RecA protease is produced as long as there are persistent DNA lesions (Bockrath and Hanawalt 1980; Theile et al. 1981). It may also be that induced RecA protein bound along single stranded DNA in oligomeric forms (West et al. 1980) cannot cleave repressors (Quillardet et al. 1981).

Taken together, these results point to the following model. It has been suggested that the F factor transferred from a UV-irradiated donor consists of the dense transferred DNA strand containing UV-induced pyrimidine dimers and a new strand synthesized in the recipient cell which is discontinuous at each lesion (Howard-Flanders et al. 1968; George and Devoret

1971; Willetts and Skurray 1980). Similar "gaps" are produced by replication of a UV-damaged chromosome (Rupp and Howard-Flanders 1968). Such "gaps" very likely provide the sites where RecA protein is activated (Craig and Roberts 1980). Indeed, a complex may be formed composed of at least a single-stranded DNA region, RecA protein, dATP, and sensitive repressors (Phizicky and Roberts 1981). Formation of such a complex may be the critical regulatory event in "indirect" as well as "direct" induction.

What is the rate limiting factor for activation of RecA protein ? Among the limiting factors to be considered, the extent of damaged DNA and the cellular concentration of dATP or of RecA protein, damaged DNA appears to be the most important. In effect, cleavage of prophage λ repressor does not require full induction of RecA protein synthesis (Moreau et al. 1980a), and there is a five-fold increase in dATP concentration after inducing treatments (Fangman and Novick 1968). We found that the efficiency of indirect induction of RecA protein synthesis increased roughly with the amount of damaged DNA transferred by infection with irradiated phage λoriF. The idea that the amount of damaged chromosomal DNA is also rate-limiting in direct induction is consistent with the UV dose-response relationship found as a function of the cellular level of λ repressor (Baluch and Sussman 1978; Bailone et al. 1979). Optimal proteolytic activity of RecA protein in vitro appears to occur when single-stranded DNA just saturates RecA protein (Craig and Roberts 1980) and other DNA binding protein such as single-strand DNA-binding (SSB) protein

(Weinstock et al. 1981). However, if SSB protein is required for the maintenance of single-strand gaps formed during replication of damaged DNA (Rupp and Howard-Flanders 1968; Baluch et al. 1980), no interaction between SSB and RecA protein appears necessary for proteolysis (Phizicky and Roberts 1981; Weinstock et al. 1981).

On the other hand, our results suggest that SOS genes have evolved so as to have various susceptibilities to RecA protease and/or to LexA repressor. The recA gene is induced at a low UV-dose even when the majority of the cells will survive, in contrast the umuC gene, which governs "inducible error-prone repair" and thus mutagenesis (Steinborn 1979; Quillardet et al. 1981), is fully induced at a relatively high UV-dose (Witkin 1976). If LexA protein is the repressor of both recA and umuC genes (Kenyon and Walker 1980; Quillardet et al. 1981), the different affinity of LexA repressor for the operators of these SOS genes should play a regulatory role (Brent and Ptashne 1981). It is our contention that as a function of DNA damage produced, more and more SOS genes are induced. Prophage λ induction occurs at a high dose when there is almost no chance for the cell to survive.

Why phage λ fails to trigger indirect induction (Rosner et al. 1968) ? The finding that UV-damaged λoriF induces prophage ϕ80 (Devoret 1981) and RecA protein synthesis upon infection (see Fig. 10) eliminates a possible role of the number of DNA strands (single or double) transferred to the recipient. We favor the following explanation : the gaps produced in phage DNA might be repaired rapidly by recombinational

events as it occurs upon multiplicity reactivation of phage (see review by Bernstein 1981). Thus, DNA lesions will not remain as such long enough to permit sufficient repressor cleavage for the derepression of recA or λ genes. Lack of recA gene induction by a UV-irradiated Hfr might also be accounted for by rapid recombinational events induced by DNA lesions. It has been shown that the leading region of the Hfr chromosome undergoes pairing with the homologous segment of the recipient chromosome before further transfer can take place (Paul and Riley 1974).

In conclusion, our results not only support the model that damaged (gapped) DNA is the limiting factor in the activation of the RecA protein-dATP complex to cleave specific cellular repressors, they also indicate that the structure of the damaged DNA is crucial. In effect, UV-damaged λoriF produced indirect induction whereas damaged λ of Hfr were poorly if not at all active. Since de novo protein synthesis is required for cleavage of λ repressor, conversion of RecA protein into a protease might need the action of other factors such as products of genes implicated in the regulation of DNA replication. Further study with mutants of phage λoriF might reveal the nature of such factors.

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LEGENDS TO TABLES

Table 1 : Bacterial strains

The alias provides the Gif member of strains from other laboratories.

Table 1

Bacterial strains

Strains	Chromosomal markers used	Plasmids or prophages	Origin or source and alias
AB1157		F^-	Howard-Flanders and Boyce 1966 ; GY752
AB1886	uvrA6	F^-	Howard-Flanders and Boyce 1966 ; GY753
AB3113	recA13	Hfr313	Howard-Flanders, GY781
CSH7	lacY1 rpsL	F^-	Miller 1972 ; GY2707
CSH23	Δ(lac pro)	$Flac^+$ $proA^+B^+$	Miller 1972 ; GY2723
GY569	uvrB501 recA13 lacY1	F^-	rec^- recombinant of KMBL90 x AB3113
GY3639	uvrA16	(λ)	Levine et al. 1979
GY3952	uvrB501 recA13 lacY1	$Flac^+$ $proA^+B^+$	GY569 x CSH23
GY3954	recAΔ21 spr-51 lexA3	F^-	GY4915
GY3956	recAΔ21 spr-51 lexA3	$Flac^+$ $proA^+B^+$	GY3954 x CSH23
GY3958	recA1 Mal^- Lam^R	Hfr KL16	KL16-99 (Low 1968)
GY3959	uvrB501 recA13 lacY1 Mal^- Lam^R	$Flac^+$ $proA^+B^+$	GY3952
GY4816	uvrA16	pKB202	Levine et al. 1979
GY4915	recAΔ21 spr-51 lexA3	F^+	Moreau et al 1980b
KMBL90	uvrB501 lacY1	F^-	GY169
W1177	lacY1 lamB	(λ)	Borek and Ryan 1958 ; GY2142

LEGENDS TO FIGURES

Fig. 1 : Kinetics of indirect induction of prophage λ

Donor bacteria GY3959 : uvr⁻recA⁻/F'lac⁺pro⁺grown in B medium up to an OD = 0.2 were centrifuged, resuspended in 1/8 the volume of cold A medium, and exposed to a UV-dose of 80 J/m^2 . The control culture was treated as above except for UV-irradiation. Recipient lysogenic bacteria W1177 :(λ)grown in B medium up to an OD = 0.2 were washed twice in cold A medium to reduce the level of free phage due to spontaneous production. Equal volumes of irradiated(*) (●) or non-irradiated (▲) donor and recipient cell suspensions were mixed for mating, A medium was complemented to reconstitute the original growth medium, and the mixtures were incubated with gentle agitation. After 30 min of incubation, a sample of each culture was treated by vigorous shaking, diluted 10-fold in prewarmed B medium and streptomycin was added to a final concentration of 100 µg/ml to prevent further mating. The cell suspensions were further incubated with aeration and at the times indicated on the abscissa, 0.1 ml samples were assayed for plaque forming units as previously described (Moreau et al . 1976). GY3959* : F-lac recA⁻ x W1177 : (λ) (●) ; GY3959 : F-lac recA⁻ x W1177 : (λ) (▲).

Fig. 2 : Kinetics of indirect induction of RecA protein synthesi

Donor bacteria GY3952 : uvr⁻recA⁻/F'lac⁺pro⁺grown in B medium up to an OD = 0.2 were centrifuged, resuspended in 1/8 the volume of cold A medium and exposed to a UV-dose of 80 J/m^2.

As a control, F^- parental bacteria GY569 : *uvr*$^-$ *recA*$^-$ were prepared as described above. Recipient bacteria GY4816 : *uvr*$^-$ *recA*$^+$/pKB202 grown in B medium up to an OD = 0.2 were centrifuged, the pellet was resuspended in the same volume of cold A medium, and half of the cell suspension was exposed to a UV-dose of 10 J/m^2. Equal volumes of cell suspension were mixed as follows. Mixture A : GY569* (F^- *recA*$^-$) + GY4816* (F^- *recA*$^+$) ; B : GY3952* (F-*lac* *recA*$^-$) x GY4816 (F^- *recA*$^+$) ; C : GY569* (F^- *recA*$^-$) + GY4816 (F^- *recA*$^+$). Each "mating" mixture was complemented to reconstitute B medium and incubation was continued with gentle agitation. At the times indicated, 1 ml samples were pulse-labeled with ^{35}S-methionine (N.E.N., 20 µCi/ml, 3 Ci/mmol) for 3 min (the incorporation of ^{35}S counts was linear for at least 6 min). Labelling was stopped by adding 0.4 ml cold YM9 containing 500 µg/ml methionine and 10 mM NaN_3. Bacteria were processed for RecA protein immunoprecipitation and SDS-PAGE as described in Materials and Methods. The band of RecA protein is indicated by an arrow on the autoradiogram.

Fig. 3 : Kinetics of RecA and total protein syntheses

The amount of RecA protein measured on the autoradiogram (▲) (Fig. 2) by densitometer tracing of the RecA band was taken as a measure of the rate of synthesis of RecA protein at the time of the pulse-labeling. Aliquots of the crude extracts prepared to measure RecA protein were precipitated with 10 % TCA. Total methionine incorporation was taken as a measure of the rate of total protein synthesis (●). A : GY569* (F^- *recA*$^-$) + GY4816*(F^-*rec*
B : GY3952* (F-*lac* *recA*$^-$) x GY4816 (F^-*recA*$^+$) ; C : GY569*(F^-*recA*$^-$)+
GY4816 (F^-*recA*$^+$).

Fig. 4 : Amount of RecA protein synthesized after indirect induction.

The cell mixtures were prepared as described in Fig. 2. After 30 min incubation with gentle agitation to allow mating, $H_2{}^{35}SO_4$ (N.E.N., specific activity 25 mCi/ml) was added to a final concentration of 200 µCi/ml and cell suspensions were further incubated with vigorous agitation for 25 min (incorporation of ^{35}S-counts was linear for 80 min). Cold $MgSO_4$ was then added to a final concentration of 10mM and the cell suspensions were incubated for a further 5 min. Samples (0.7 ml) were mixed with 0.3 ml cold YM9 containing 10 mM NaN_3 , and processed for specific RecA protein precipitation and SDS-PAGE. a : GY569* (F^- *recA*$^-$) + GY4816 (F^- *recA*$^+$) ; b : GY4816 (F^- *recA*$^+$) ; c : GY3952* (F-*lac* *recA*$^-$) x GY4816 (F^- *recA*$^+$) ; d : GY3952* (F-*lac* *recA*$^-$) ; e : immunoprecipitate material obtained from strain KM1842, which synthesizes large amounts of RecA protein ; the band of RecA protein is indicated by an arrow on the autoradiogram (Moreau *et al*. 1980).

Fig. 5 : Measurement of RecA protein by "rocket" immununoelectrophoresis.

Donor bacteria GY3956 : *recA*Δ*21*/F' *lac*$^+$*pro*$^+$ (or F^- parental bacteria GY3954) grown in B medium up to an OD = 0.2 were centrifuged, resuspended in 1/3 the volume of cold A medium and exposed to a UV-dose of 80 J/m^2 . Recipient bacteria GY4816 : *uvr*$^-$/pKB202 were prepared as described in Fig. 2. Equal volumes of cell suspensions were mixed and restored to the original growth medium. Additional incubation was with gentle agitation for 30 min ; and then with vigorous agitation for 50 min ;

14 ml samples were then mixed with 3 ml of cold YM9 containing 10 mM NaN_3, and processed for rocket immunoelectrophoresis. Wells c-g : GY3956 (F-lac Δ recA) + GY4816 (F^- $recA^+$) (zero time) ; GY3954* (F^- ΔrecA) + GY4816* (F^- $recA^+$) ; GY3954* (F^- ΔrecA) + GY4816 (F^- $recA^+$) ; GY3956 (F-lac ΔrecA) x GY4816 (F^- $recA^+$) ; GY3956* (F-lac ΔrecA) x GY4816 (F^- $recA^+$) . Wells a and b contained respectively 2.5 and 1.25 µg of purified RecA protein.

Fig 6 : Direct induction of RecA protein synthesis

Bacteria GY4816 : uvrA16 or AB1157:uvr^+ grown in B medium up to an OD = 0.2 were centrifuged, resuspended in the same volume of cold A medium and exposed to UV-light, respectively 10 and 80 J/m^2. The original growth medium was reconstituted and incubation was continued with vigorous agitation. At the times indicated, 20 ml samples were mixed with 4 ml cold YM9 containing 10 mM NaN_3 and further processed for rocket immunoelectrophoresis. The zero time was taken just before irradiation.

Fig. 7 : Kinetics of direct induction of RecA protein synthesis

Bacteria AB1157 : uvr^+ (o) and AB1886 : uvrA6 (Δ) were grown in enriched YM9 medium up to an OD = 0.15 and chilled. Samples of 20 ml were exposed to increasing UV-doses, further incubated for 30 min at 37°C, mixed with 10 ml cold YM9 containing 10 mM NaN_3, and further processed for rocket-IEP. Immediately after UV-irradiation, 0.1 ml samples were taken to measure cell survival. Cell survival (panel A) and cellular level of RecA protein (panel B) are plotted versus UV-doses.

Fig. 8 : Indirect induction of λ repressor cleavage

Bacteria GY4816 : uvr⁻/pKB202 or GY3639 : uvr⁻ (λ) were grown in low sulfur medium (C) containing 0.4 mCi/ml $H_2{}^{35}SO_4$ from an OD = 0.05 to an OD = 0.2 before the addition of 10 mM cold $MgSO_4$. The cultures were incubated for a further 5 min, pelleted by centrifugation, resuspended in the same volume of cold A medium, and irradiated (10 J/m²) when required. Donor bacteria GY3952 : uvr⁻rec⁻/F-lac⁺pro⁺ (or F⁻ parental bacteria GY569) were grown, irradiated as described in Fig. 2, and mixed (v/v) with GY4816 or GY3639. Complement was added to reconstitute D medium. Incubation was with gentle agitation for 30 min and then with vigorous agitation for 30 min ; 1 ml samples were then mixed with 0.4 ml cold YM9 containing 10 mM NaN_3, and processed for specific repressor precipitation and SDS-PAGE. Autoradiograms and densitometer tracing of autoradiograms are shown. a : GY569* (F⁻) + GY4816* (/pKB202) ; b : GY569* (F⁻) + GY4816 (/pKB202) ; c : GY3952 (F-lac) x GY4816 (/pKB202) ; d : GY3952* (F-lac) x GY4816 (/pKB202) ; e : GY569* (F⁻) + GY3639 (λ) ; f : GY3952 (F-lac) x GY3639 (λ) ; g GY3952* (F-lac) x GY3639 (λ).

Fig. 9 : UV-dose dependent induction of RecA protein synthesis by phage λoriF.

Bacteria GY4816 : uvr⁻/pKB202 were grown in B medium up to an OD = 0.2 and chilled on ice. Phage λoriF exposed to UV-light (0 - 160 J/m²) were mixed, at a multiplicity of 10, with 7 ml of bacteria and allow to adsorb for 10 min. The suspensions were incubated with gentle agitation for 30 min, with vigorous agitation for further 50 min and then mixed with cold YM9

containing 10 mM NaN_3, and processed for rocket immunoelectrophoresis. Wells a - f : 0, 20, 40, 80, 120 and 160 J/m^2 to phage λoriF. Wells g - j : 0.625, 1.25, 2.5 and 5 µg of purified RecA protein.

Fig. 10 : The concentration of RecA protein per assay was calculated as the ratio of the amount of RecA protein measured on Fig. 9 to the amount of total protein measured by the Bio-Rad protein assay procedure. The concentration of RecA protein normalized to the background level of non-irradiated bacteria was plotted versus the dose of irradiation to phage λoriF.

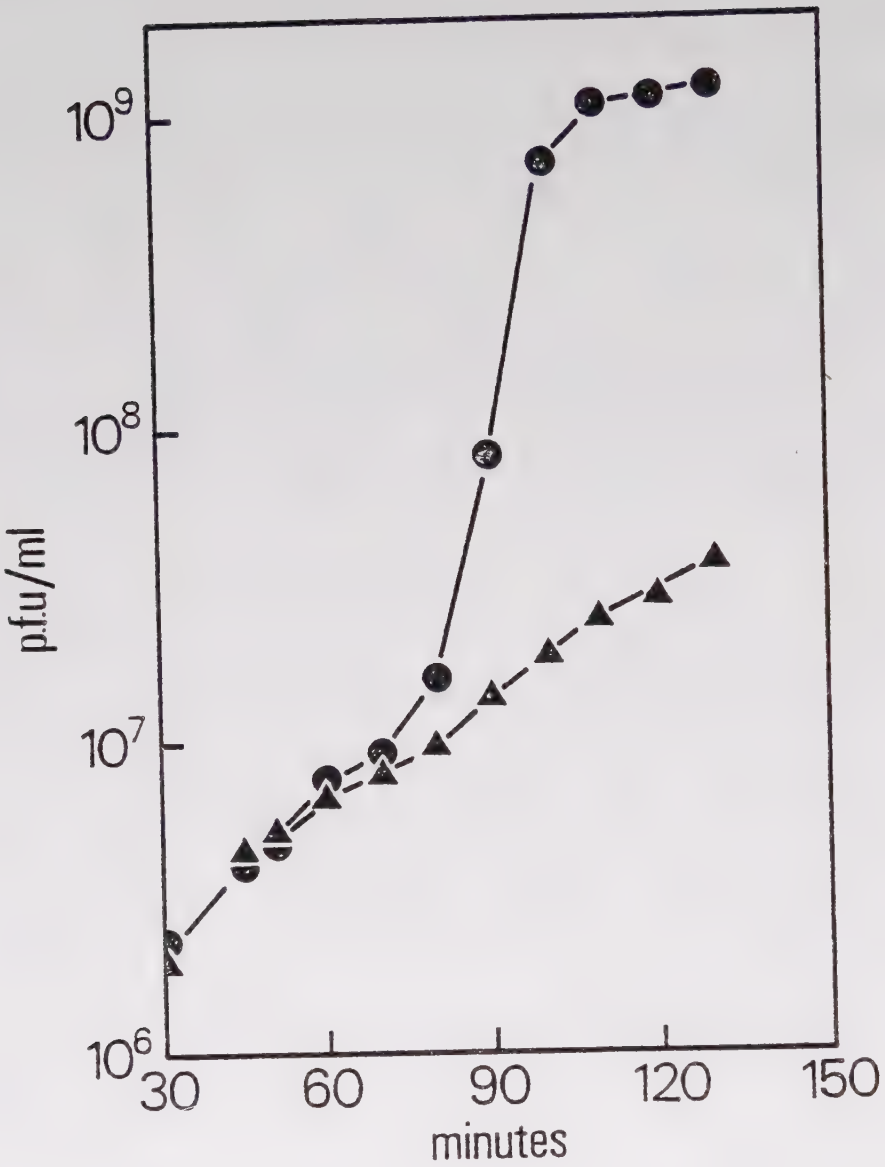

Fig.1

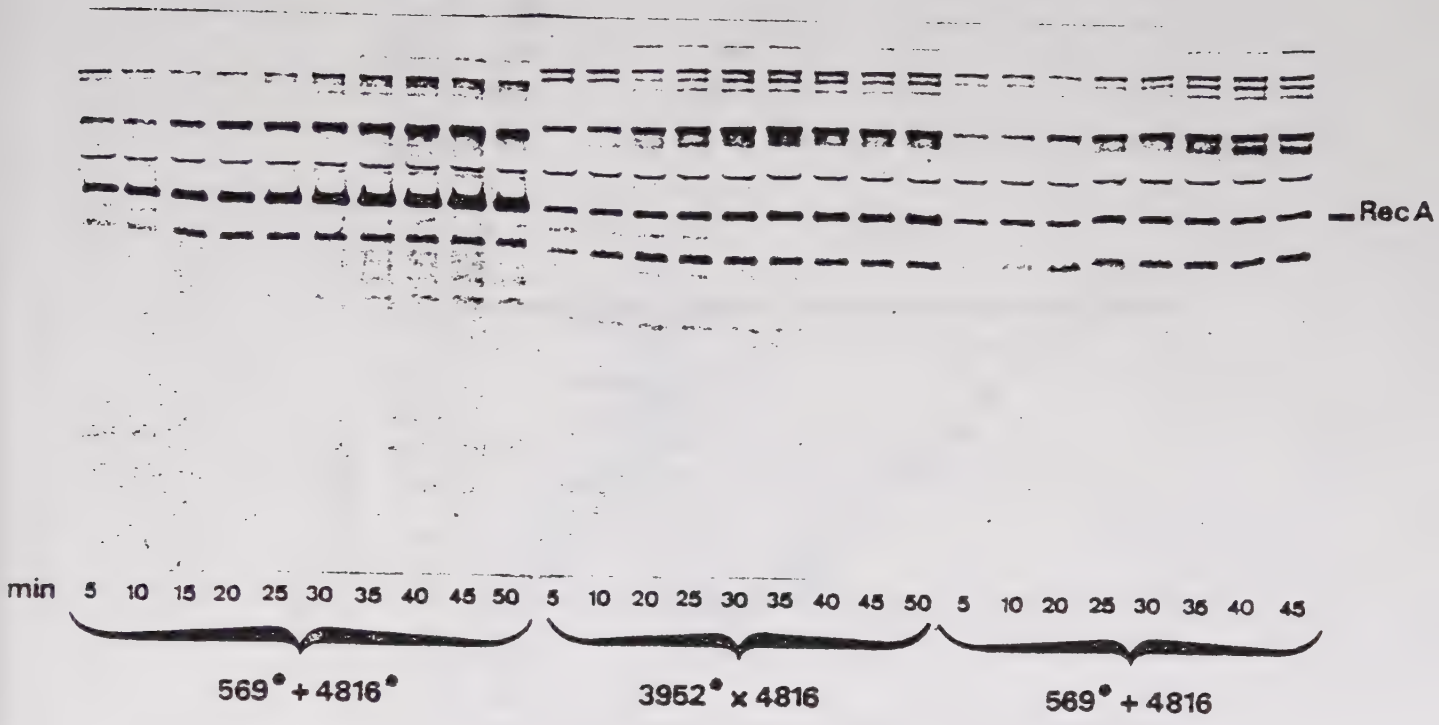
RecA
min 5 10 15 20 25 30 35 40 45 50 5 10 20 25 30 35 40 45 50 5 10 20 25 30 35 40 45
569* + 4816*
3952* x 4816
569* + 4816

Fig.2

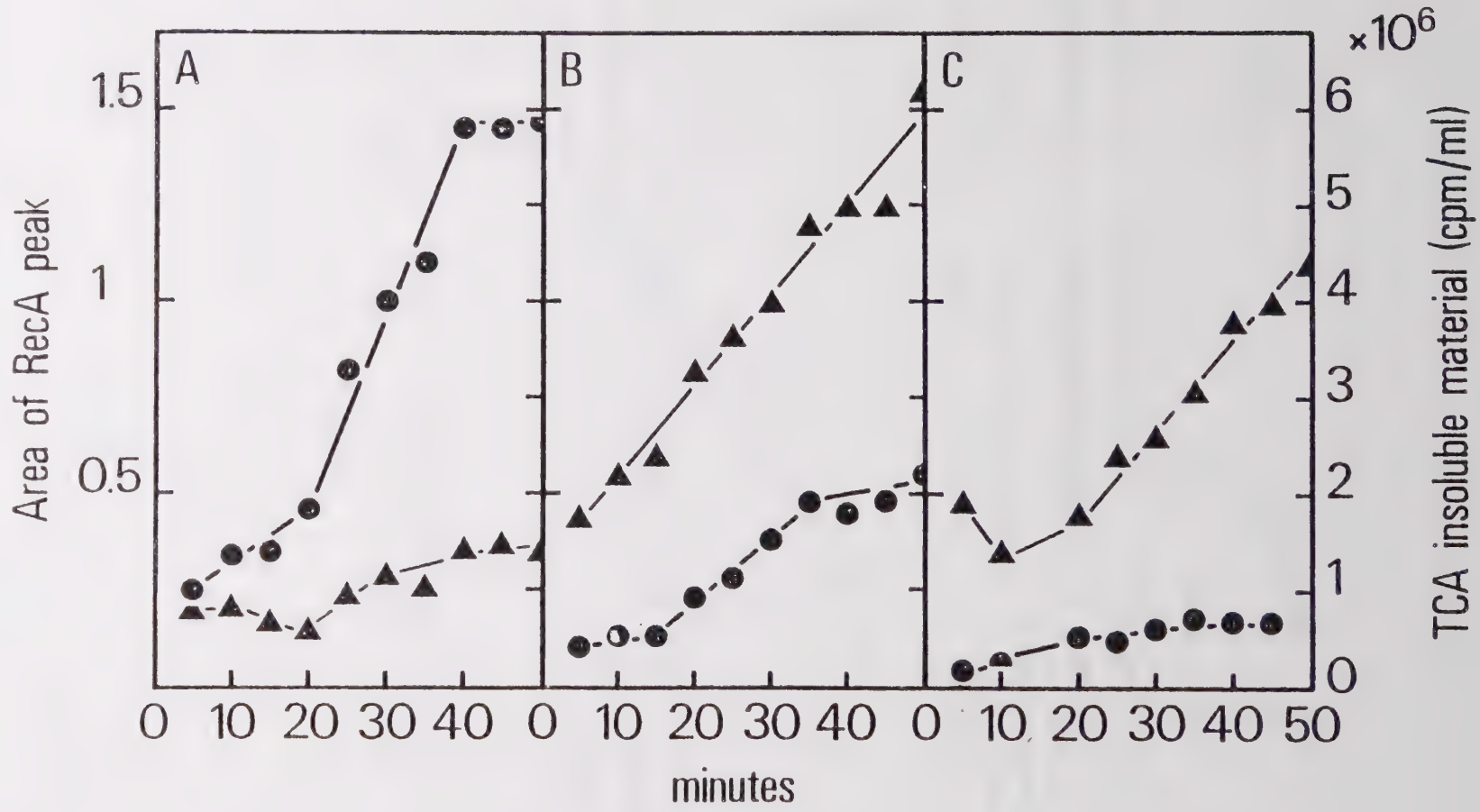
A
B
C
1.5
1
0.5
Area of RecA peak
×10^6
6
5
4
3
2
1
0
TCA insoluble material (cpm/ml)
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40 50
minutes

Fig. 3

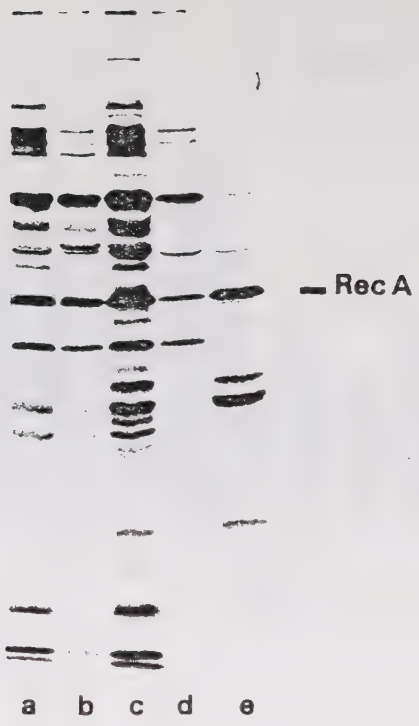

Fig.4

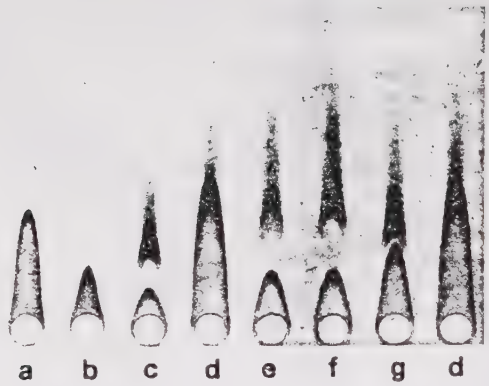

Fig.5

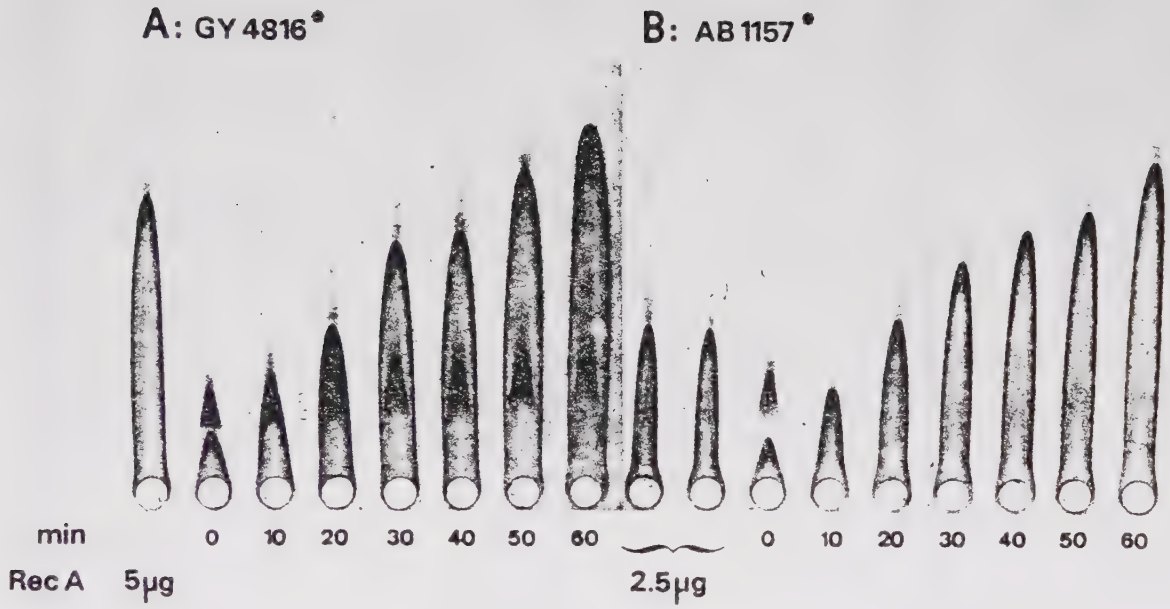
A: GY 4816*
B: AB 1157*
min
0 10 20 30 40 50 60
0 10 20 30 40 50 60
Rec A
5µg
2.5µg

Fig. 6

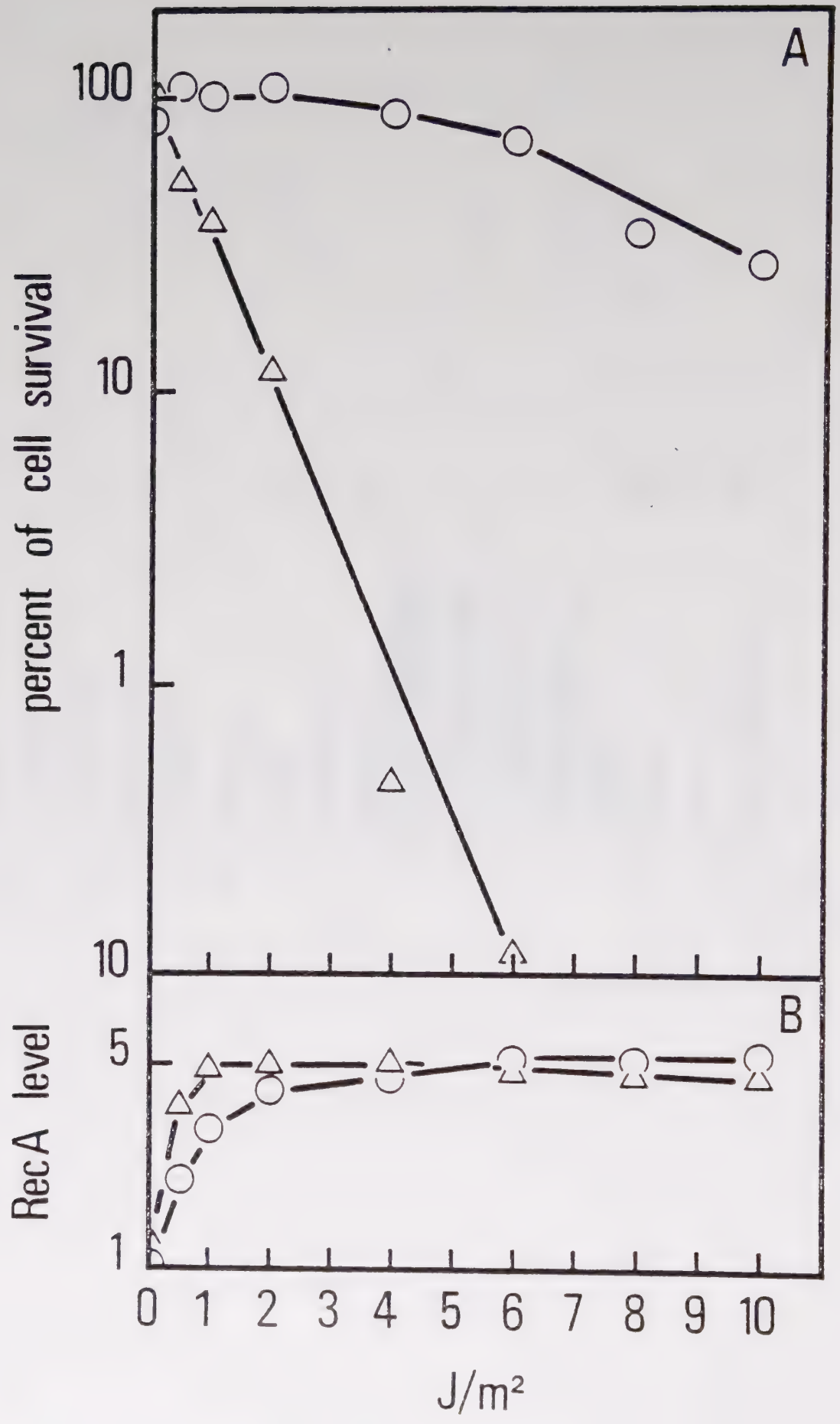
A
100
10
1
10
percent of cell survival
B
5
1
RecA level
0 1 2 3 4 5 6 7 8 9 10
J/m²

Fig.7

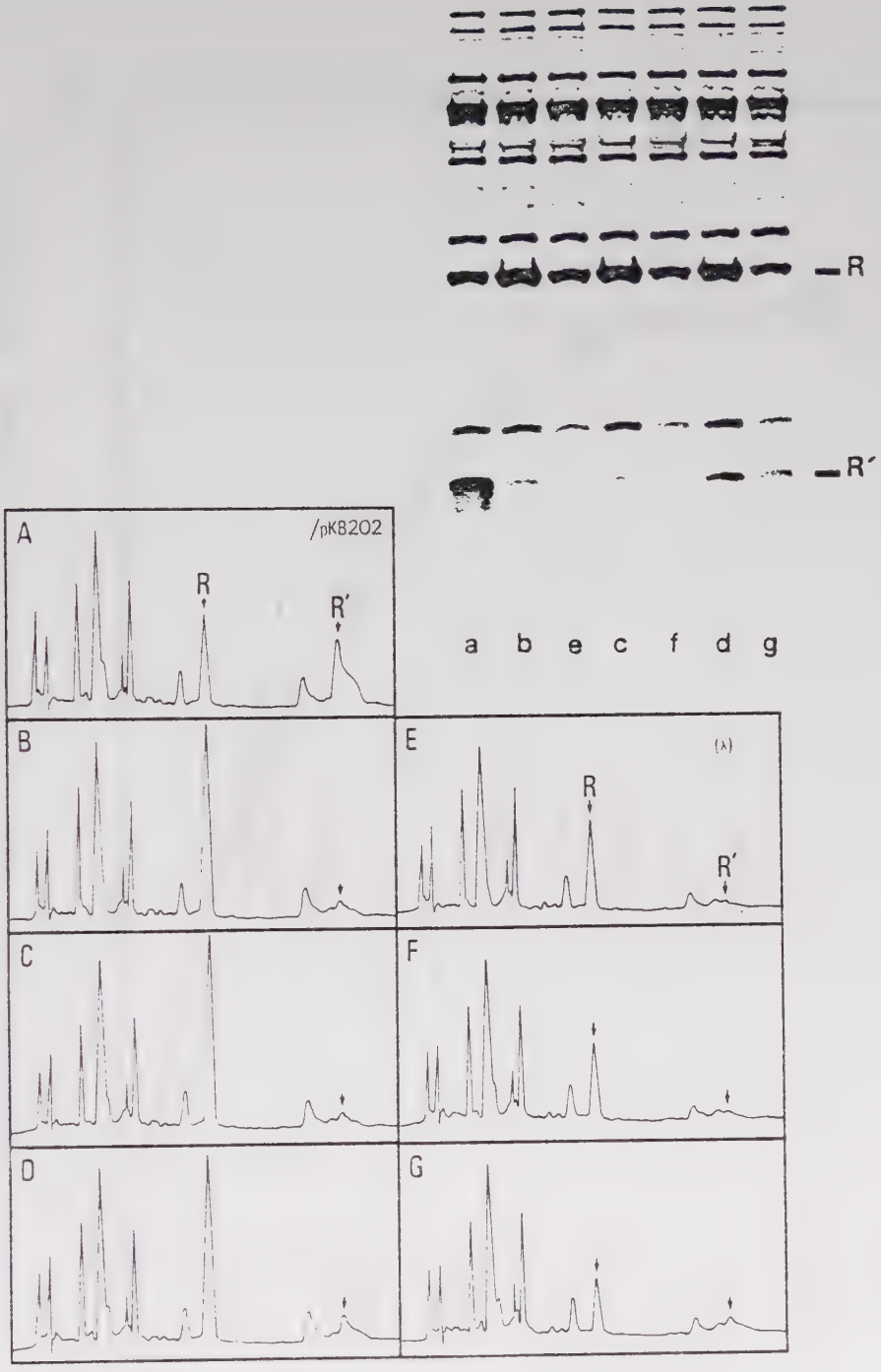

Fig.8

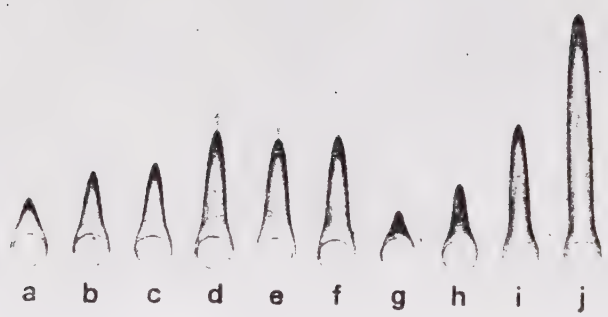

Fig.9

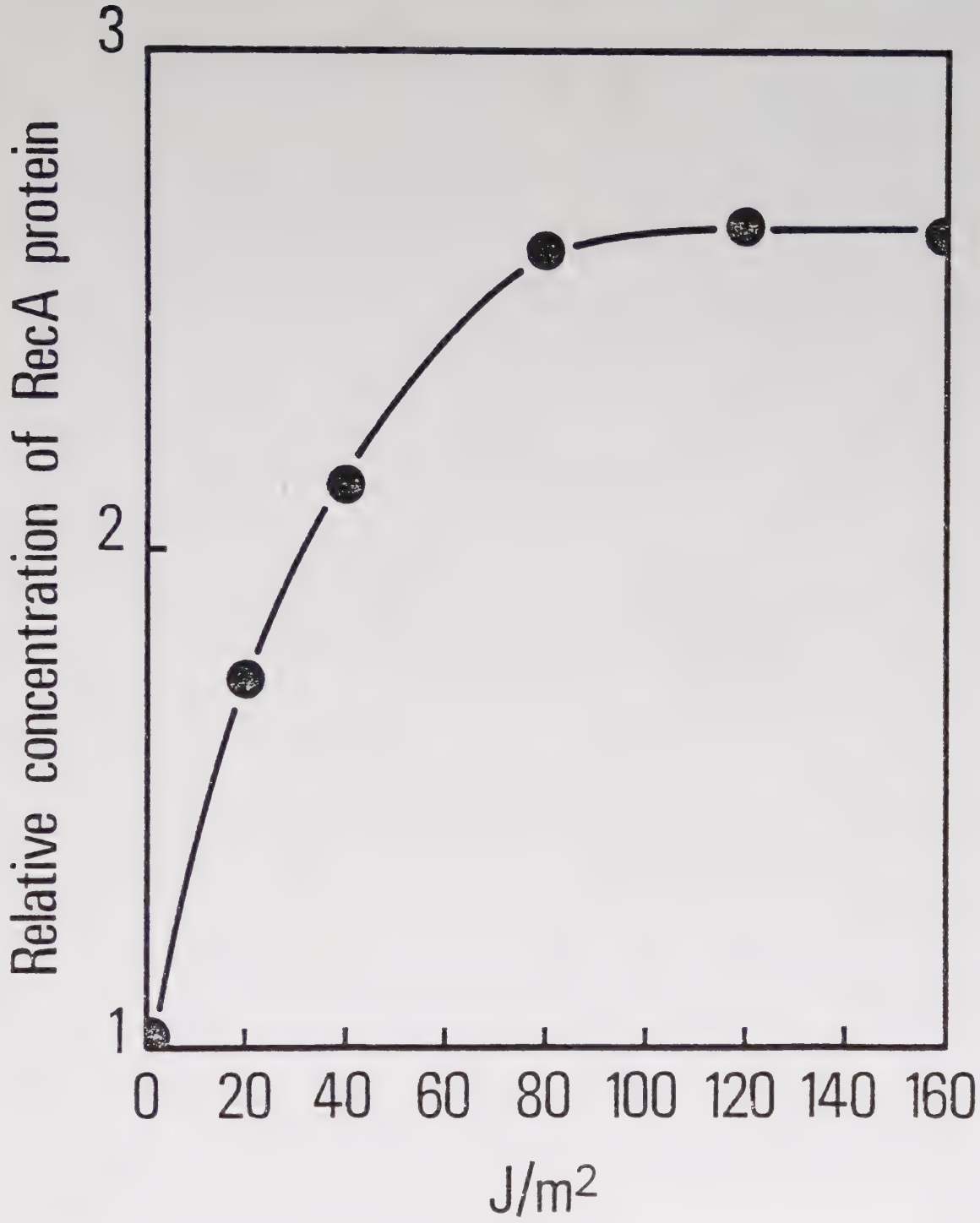
Relative concentration of RecA protein
3
2
1
0
20
40
60
80
100
120
140
160
J/m2

Fig.10

D I S C U S S I O N G E N E R A L E

MECANISMES DE LA MUTAGENESE ET DE L'INDUCTION LYSOGENIQUE. PRINCIPE DES TESTS BACTERIENS POUR LA DETECTION DES CANCEROGENES ET ANTITUMORAUX POTENTIELS

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RESUME

Les données génétiques et biochimiques récentes concernant les processus déclenchés dans une bactérie dont l'ADN a été endommagé sont résumées dans cet article. On met l'accent sur la différence qui existe entre les mutagènes "directs" (type éthylméthanesulfonate) et les mutagènes "indirects" (type rayons ultraviolets). Ces derniers agents induisent en effet différents évènements cellulaires, communément appelés "fonctions SOS", parmi lesquels nous citerons : la réparation mutagène de l'ADN, la filamentation et l'induction lysogénique. Nous proposons un modèle moléculaire qui rend compte de l'induction séquentielle de ces différentes fonctions SOS.

Mots clés

Bactéries, bactériophages, mutagénèse, induction lysogénique, fonctions SOS.

SUMMARY

Recent genetic and biochemical data concerning the cellular responses to DNA damage in bacteria emphasize the difference which exists between "direct" and "indirect" mutagens.

Direct mutagens, such as ethyl methanesulfonate, provoke erroneous base pairing (mispairing) during DNA replication. This results in specific base-pair substitutions.

Indirect mutagens, such as UV-light or mitomycin C, impede the progression of the replication fork. This event leads to the induction of various cellular phenomena called SOS functions. SOS functions include a rapid and massive synthesis of RecA protein, inducible error-prone repair, inhibition of cellular division, and activation of inducible prophages (lysogenic induction). A model that accounts for the coordinate induction of these SOS phenomena is proposed.

Key words :

Bacteria, bacteriophages, mutagenesis, lysogenic induction, SOS functions.

Introduction

Au cours des cinq dernières années, de nombreux tests bactériens ont été proposés pour détecter, parmi les substances présentes dans notre environnement, celles qui pourraient présenter chez l'homme des activités cancérogènes ou, plus généralement, des activités mutagènes. Parmi ces tests, dits à "court terme", ceux qui sont le plus couramment utilisés (1, 13, 25) sont basés sur la détection de l'un des deux phénomènes suivants :

1) un phénomène génétique : la mutagénèse.

2) un phénomène épigénétique : l'induction lysogénique. Des virus, ou bactériophages, qui sont normalement maintenus à l'état dormant dans des bactéries dites lysogènes, sont induits à se développer de façon autonome quand l'ADN des cellules qui les abritent est endommagé.

Ces deux phénomènes présentent de nombreuses analogies, mais aussi des différences riches d'enseignement pour préciser l'action d'une substance chimique sur l'ADN.

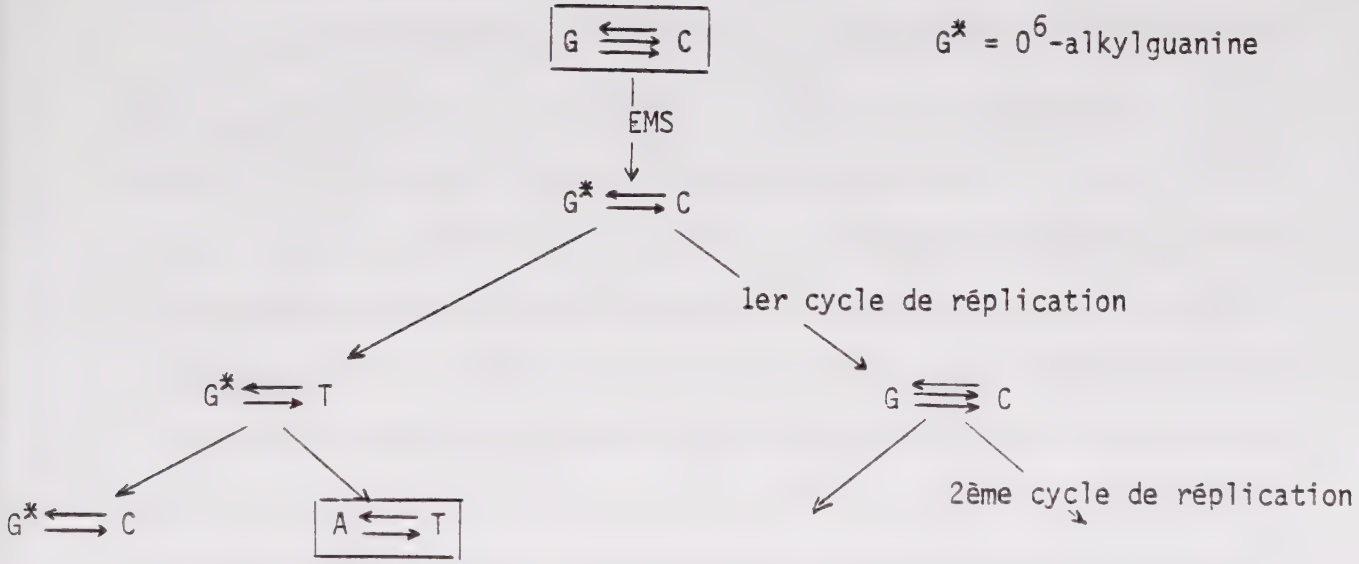
Mutagénèse "Directe" ou "Indirecte"

Quand des agents physiques (rayons ultraviolets, rayons X) ou chimiques (éthylméthanesulfonate, mitomycine C) endommagent le matériel génétique d'une bactérie (ADN), différents systèmes de réparation sont immédiatement mis en jeu (17, 18, 23, 24, 28, 53). Ces systèmes de réparation, qui opèrent généralement par excision des bases ou des nucléotides altérés, sont essentiellement "fidèles", c'est à dire qu'ils reconstituent la molécule d'ADN sans modifier l'information qu'elle contenait. Toutefois, si ces systèmes de réparation sont débordés par le nombre de

lésions, certaines d'entre elles persistent sur un ADN qui se réplique. Deux cas sont alors envisageables (Figure 1) :

1) Les altérations de l'ADN n'empêchent pas la réplication de se produire, mais elles introduisent des erreurs de codage (17, 18).

L'exemple le mieux connu est celui des bases alkylées par un agent tel que l'éthylméthanesulfonate (EMS). L'EMS alkyle en particulier la guanine (une des quatre bases qui entrent dans la composition de l'ADN) en position O^6. La guanine ainsi modifiée (O^6-alkylguanine) s'apparie indifféremment avec la cytosine, son partenaire normal, ou avec la thymine. Après deux cycles de réplication, on peut donc observer la substitution d'une paire de bases par une autre (transition G-C → A-T) selon le mécanisme suivant :



Dans certains cas particuliers (agents intercalants), il peut y avoir délétion (ou addition) d'un certain nombre de paires de bases : il se produit alors un décalage du cadre de lecture des bases.

Ces mécanismes de mutagénèse, qui peuvent s'exprimer chez des bactéries portant les mutations recA ou lexA, ont été regroupés sous le terme générique de mutagénèse directe, ou mutagénèse par mauvais appariement des bases ("direct", "mispairing", ou "misreplication mutagenesis") (29, 46).

2) Les lésions de l'ADN empêchent la progression normale de la fourche de réplication. Ces lésions, dites "non-codantes", résultent le plus souvent de la fixation covalente d'une substance chimique sur l'ADN, ou de l'induction de cassures d'ADN simple brin par un mécanisme radicalaire. En fait, le cas le mieux étudié au niveau moléculaire est celui, apparemment très particulier, du rayonnement ultraviolet (57).

Les rayons ultraviolets (UV) (254 nm) ont pour cible principale l'ADN, chez qui ils provoquent essentiellement la formation de dimères entre pyrimidines contigües sur le même brin ($\widehat{TT}$, $\widehat{CT}$). Il semble qu'un dimère ne puisse pas être répliqué (46). Puisque la réplication se fait de façon discontinue sur les deux brins d'ADN (41), on peut concevoir aisément que le complexe de réplication stoppe la polymérisation des nucléotides au niveau d'un dimère et "glisse" jusqu'au prochain site de réinitiation d'un "fragment d'ADN naissant". Un "vide" ("gap") est ainsi laissé en arrière de la fourche de réplication (51). Ce type de dommage peut être réparé par recombinaison entre le brin d'ADN nouvellement synthétisé et le brin d'ADN parental de même polarité (réparation post-réplicative par recombinaison). Le matériel génétique ainsi échangé aurait une longueur au moins égale à 1000 nucléotides (52) et pourrait contenir des dimères qui seraient ainsi progressivement retirés du brin parental

irradié (19). Comme dans le cas de la réparation par excision, ce mécanisme de réparation serait essentiellement fidèle et constitutif, c'est-à-dire qu'il peut se produire en l'absence de nouvelle synthèse protéique (23, 57). Toutefois, l'efficacité de ce mécanisme étant faible, des lésions non réparées pourraient constituer un "signal" pour l'induction de nouvelles fonctions cellulaires, communément appelées "fonctions SOS", puisqu'elles semblent sauver une cellule en danger (57).

Les fonctions SOS les mieux étudiées sont : l'induction de nouveaux systèmes de réparation infidèles de l'ADN et l'induction de bactéries lysogènes à produire des phages (Figure 1). Ces différentes réponses à l'endommagement grave de l'ADN sont induites par les mêmes doses d'UV ; elles requièrent pour se produire l'expression des gènes recA et lexA ainsi qu'une synthèse protéique de novo (11, 12, 14, 45, 57).

Réparation SOS

Les fonctions SOS aboutiraient en particulier à la modification du complexe de réplication (ou de certaines enzymes de ce complexe) qui pourrait alors combler les vides laissés dans le chromosome ; cette réparation se ferait au prix d'une inhibition de la fidélité de réplication (45, 56). Les erreurs commises pendant l'incorporation des nucléotides pouvant théoriquement se produire tout au long de cette synthèse de réparation, un seul dommage (un dimère) peut donc induire plusieurs mutations adjacentes. Ce type de mutagénèse, qui nécessite l'induction des fonctions SOS, a été appelé mutagénèse "indirecte" ou "induite".

Les "vides" laissés dans le chromosome constituent à la fois le signal pour l'induction des fonctions SOS et le substrat sur lequel

agissent les enzymes de réparation mutagènes. D'autres régions d'ADN monocaténaire - de larges "brèches" produites par l'excision des lésions et la dégradation partielle de l'ADN ("long patch") (23) - pourraient également jouer le rôle de substrat pour les enzymes de réparation mutagènes (6). Ce type de mutagénèse, qui résulte essentiellement de la réparation mutagène des lésions de l'ADN, est dit "avec cible" ("targeted"), par opposition à la mutagénèse induite "sans cible" ("untargeted") (58). En effet, certains agents agissent spécifiquement au niveau de la fourche de réplication ; ils induisent les fonctions SOS sans pour autant causer de lésions sur l'ensemble du chromosome. L'acide nalidixique appartient à cette catégorie de produits. Cet antibiotique inhibe une enzyme, la gyrase, dont la fonction est indispensable à la réplication du chromosome bactérien (8). Dans ce cas, la mutagénèse est très peu importante puisque le substrat sur lequel agissent préférentiellement les enzymes de réparation mutagènes (les régions d'ADN simple brin) n'est présent qu'en très faible quantité au niveau de la fourche de réplication bloquée. Toutefois, pendant le premier cycle de réplication qui suit l'induction des fonctions SOS, la fidélité de la réplication étant globalement diminuée, des erreurs d'incorporation des nucléotides peuvent se produire "spontanément", c'est-à-dire sur un ADN intact. Dans le cas de l'acide nalidixique, la mutagénèse induite résulte donc essentiellement d'une "réplication mutagène" (appelée aussi "effet mutateur"), alors que dans le cas des rayons ultraviolets, la "réparation mutagène" des lésions de l'ADN est le composant principal de l'effet mutagène.

Si le mécanisme de la mutagénèse induite n'est pas encore élucidé, par contre, celui de l'induction lysogénique est beaucoup mieux compris au niveau moléculaire.

Induction Lysogénique

Certains virus (bactériophages), dits tempérés, ont la possibilité de se reproduire en symbiose avec les bactéries qu'ils infectent. Les bactéries abritant un tel phage à l'état dormant sont dites lysogènes. Le système lysogénique le mieux étudié est constitué par le couple bactérie hôte Escherichia coli K12 - phage λ. Dans une bactérie lysogène E. coli K12 (λ), l'ADN du phage λ, ou prophage λ, est intégré dans le chromosome bactérien. Le prophage λ est ainsi transmis à l'état latent de cellule mère à cellules filles. Comme nous l'avons vu précédemment, cet état lysogène stable est interrompu brutalement quand les fonctions SOS sont induites (Figure 1). Le prophage λ est alors induit à se développer de façon autonome, ce qui conduit à la lyse de la cellule hôte et à la libération d'une centaine de particules virales par centre infectieux (induction lysogénique) (13).

Dans une bactérie lysogène, le prophage λ est maintenu à l'état dormant par l'action d'une protéine répresseur, produit du gène cI du prophage. Les molécules de répresseur se fixent sur l'ADN, probablement sous forme de dimères, au niveau de deux sites opérateurs (O_L et O_R) situés de part et d'autre du gène cI ; le répresseur bloque ainsi la transcription des gènes viraux précoces. En fait, le répresseur empêche l'accès de la RNA polymérase aux sites promoteurs (P_L et P_R) contigus aux sites opérateurs (43).

L'induction lysogénique résulte de l'inactivation du répresseur phagique (3, 54). Dans des bactéries lysogènes induites par la mitomycine C, le répresseur est clivé en deux fragments identiques (48). In vitro, la protéine RecA clive le répresseur du prophage λ (9, 10, 42, 49, 50), ce qui suggère que la destruction du répresseur est bien la cause, et non la conséquence, de son inactivation.

La synthèse de la protéine RecA est augmentée dans des cellules dont l'ADN a été endommagé (21, 22, 34). De nombreux auteurs ont donc suggéré que l'amplification de la protéine RecA pouvait être responsable de l'induction des différentes fonctions SOS. Nous avons montré que cette hypothèse est fausse en ce qui concerne l'induction du prophage λ (37).

Le clivage du répresseur du prophage λ peut se produire normalement dans des bactéries lysogènes induites par la mitomycine C, bien que la synthèse de la protéine RecA soit spécifiquement réduite par une faible concentration de rifampicine. La production de phages libres est toutefois réduite dans ces conditions (37). Il apparait donc que l'amplification de la protéine RecA est requise, non pour le clivage du répresseur du prophage λ, mais pour la réparation de l'ADN endommagé. Des résultats similaires ont été obtenus en utilisant des bactéries portant différentes mutations dans le gène lexA (lexA1 ou lexA3) ; ces mutations empêchent notamment l'induction de la synthèse de la protéine RecA (15, 44).

Ces résultats sont en accord avec l'hypothèse selon laquelle il existerait deux populations de protéine RecA dans une cellule dont l'ADN a été endommagé (Figure 2) :

1) une population majoritaire de molécules "natives" requise pour la réparation non mutagène de l'ADN. Il est probable que la protéine RecA à l'état natif se "colle" en longs filaments sur l'ADN endommagé pour en limiter la dégradation due, en particulier, à l'activité exonucléase V codée par les gènes recBC (21, 56).

2) une population minoritaire de molécules "activées" possédant une activité protéolytique.

Cette hypothèse implique l'existence de "signaux moléculaires" conférant à la protéine RecA une activité protéolytique. Ces "co-facteurs" seraient produits en quantité limitée par rapport au nombre de molécules de protéine RecA synthétisées après induction des fonctions SOS.

Récemment, Roberts et coll. (9, 10, 42, 49, 50) ont montré que le clivage du répresseur du prophage λ exige *in vitro* trois composants : la protéine RecA, un nucléoside triphosphate (ATP ou dATP) et de l'ADN simple brin. Il semble que la concentration optimale d'ADN simple brin par rapport à la concentration de protéine RecA soit de cinq à six nucléotides par monomère de protéine RecA.

Les données obtenues tant *in vivo* (39) qu'*in vitro* (42) indiquent que le facteur limitant dans la réaction de clivage est la quantité d'ADN simple brin nécessaire pour activer le complexe RecA-dATP.

Mécanisme des Fonctions SOS

Les progrès réalisés dans la compréhension du mécanisme de l'induction lysogénique ont beaucoup contribué à l'élucidation des autres phénomènes SOS.

A. La protéine RecA clive la protéine LexA, répresseur des gènes *recA* et *lexA*.

De nombreuses données génétiques ont conduit à penser que l'expression des gènes *recA* et *lexA* est normalement réprimée par la protéine LexA (4, 31, 40). Le répresseur LexA serait clivé par la protéase RecA (57).

Récemment, on a pu montrer *in vitro* que :

1) la protéine LexA inhibe spécifiquement la transcription des gènes *recA* et *lexA* (5, 33).

2) la protéine RecA clive la protéine LexA au cours d'une réaction identique à celle décrite pour le clivage du répresseur du prophage λ (32, 33).

Le fait que la protéine LexA contrôle sa propre synthèse présente deux avantages évidents :

1) la concentration cellulaire de la protéine LexA est constante chez des bactéries en croissance ;

2) La protéine LexA peut agir à nouveau très rapidement comme répresseur quand, dans une cellule où les fonctions SOS ont été induites, la production de "protéase RecA" est interrompue.

B) La protéine LexA contrôle l'expression de la mutagénèse induite.

La notion de réparation induite et mutagène, ou réparation SOS, s'est dégagée des données obtenues sur la réparation de l'ADN du phage λ.

La survie de phages λ irradiés par les UV est augmentée quand ces phages se reproduisent chez des bactéries elles-mêmes préalablement irradiées. La "réactivation" des phages s'accompagne d'une forte mutagénèse (55). Ces phénomènes, connus sous les noms de "Weigle-réactivation" et "Weigle-mutagénèse" nécessitent pour se produire une synthèse protéique de novo (57). On a interprété ce double phénomène comme reflétant l'induction, dans des cellules irradiées, d'enzymes de réparation mutagènes qui agiraient indifféremment sur l'ADN endommagé d'un phage ou sur celui de la cellule (14, 45, 57).

La cinétique de W-réactivation(et de W-mutagénèse)des phages irradiés est identique à celle de la synthèse de la protéine RecA, ce qui a conduit à penser, à tort, à une relation de cause à effet entre ces deux processus. Nous avons montré (44) que, s'il existe une bonne

corrélation entre la survie bactérienne et le niveau cellulaire de protéine RecA, indépendemment du génotype lexA, par contre, les processus de "W-reactivation" et de "W-mutagénèse" dépendent largement de l'action de la protéine lexA et non pas du niveau de protéine RecA. En effet, les cinétiques de réactivation et de mutagénèse des phages λ irradiés ne sont pas modifiées chez un mutant qui synthétise de façon constitutive une grande quantité de protéine RecA (mutant opérateur constitutif recAO^c). Ce résultat indique clairement que la concentration cellulaire de protéine RecA n'est pas le facteur limitant de ces processus. De plus, il est apparu que les processus de "W-reactivation" et de "W-mutagénèse" ne s'expriment pas chez des bactéries qui portent la mutation lexA3 et ce, même si la protéine RecA est présente à un haut niveau cellulaire (mutant lexA3 recAO^c). Le double mutant lexA3 recAO^c est largement résistant aux UV contrairement au mutant d'origine lexA3. Le gène muté lexA3 codant pour une protéine qui est difficilement clivable par la protéase RecA (32), ces résultats sont en accord avec l'hypothèse selon laquelle le répresseur LexA contrôlerait, outre le gène recA, d'autres gènes impliqués notamment dans la réparation mutagène de l'ADN (umuC) (26, 27). Quand les fonctions SOS sont induites, le clivage de la protéine LexA par la protéase RecA déclencherait l'induction de ces différents gènes de façon coordonnée.

Il faut souligner que si la réparation SOS n'a qu'une importance mineure pour la survie d'une cellule (44),ce processus joue toutefois un rôle fondamental dans les processus d'adaptation de l'espèce en augmentant, de façon transitoire, le taux de mutagénèse.

Mécanisme des Fonctions SOS

Les données génétiques et biochimiques concernant le mécanisme des fonctions SOS peuvent être résumées de la façon suivante :

1) Dans une bactérie en phase exponentielle de croissance (Figure 3), la protéine LexA contrôle négativement : a) sa propre synthèse, b) l'expression du gène recA, c) l'expression d'autres "gènes SOS", notamment ceux impliqués dans la réparation de l'ADN ; cette réparation peut être essentiellement non mutagène (uvrA-dépendente) ou mutagène (umuC-dépendente) (27).

La synthèse de la protéine RecA n'est pas pour autant complètement bloquée puisqu'il existe de façon constitutive à peu près 5.000 molécules de protéine RecA par cellule (C. Paoletti, comm. pers., 39).

2) Si la bactérie est exposée à des agents qui endommagent gravement l'ADN (Figure 4), la protéine RecA vas se fixer sur les régions d'ADN simple brin qui sont formées essentiellement au niveau de la fourche de réplication : la protéine RecA acquiert une activité protéolytique contre des répresseurs cellulaires spécifiques.

Dans le cas du gène recA, le clivage de la protéine LexA rend accessible à la RNA polymérase un nouveau site promoteur plus efficace que celui utilisé en présence de la protéine LexA. La synthèse de la protéine RecA augmente alors très rapidement.

Si la bactérie est lysogène pour le prophage λ, le répresseur du prophage est également clivé par la protéase RecA ; la survie du phage λ se fait alors au détriment de celle de la cellule hôte.

3) Quand, dans une fraction de la population bactérienne, les lésions de l'ADN ont pû être réparées, la réplication normale reprend. Il s'ensuit que le signal inducteur des fonctions SOS et donc l'activité

protéolytique de la protéine RecA disparaissent. La protéine LexA peut à nouveau s'accumuler et réprimer les "gènes SOS". Les cellules survivantes qui portent maintenant un certain nombre de mutations peuvent revenir à un état de croissance normale.

4) Contrairement à ce qui a été suggéré par Witkin (57), il est évident que toutes les fonctions SOS ne sont pas induites de façon simultanée dans des bactéries de type sauvage.

Si la mutagénèse (et le développement du prophage λ) est induite à des doses d'irradiation UV fortes,
par contre, la synthèse optimale de protéine RecA se produit à des doses d'irradiation faibles auxquelles survivent la majorité des cellules (39).

Ce résultat n'est pas en contradiction avec l'hypothèse selon laquelle un même répresseur, la protéine LexA, régulerait ces deux phénomènes. Little et al. (33) et Brent et Ptashne (5) ont montré récemment que la protéine LexA s'attache plus fortement à l'opérateur du gène recA qu'à l'opérateur du gène lexA. On peut imaginer que la constante d'affinité répresseur-opérateur soit encore supérieure dans le cas du gène umuC.

Il apparaît donc que l'induction des fonctions SOS se produit de façon séquentielle et que ce processus peut être interrompu dès que les lésions de l'ADN disparaissent. Des répresseurs de nature différente peuvent avoir des "sensibilités" très variables vis-à-vis de la protéase RecA et être clivés à des vitesses différentes comme cela a été suggéré dans le cas de répresseurs phagiques (15) ; dans le cas d'un même type de répresseur, cette variabilité dépend des structures des régions opérateurs avec lesquels le répresseur interagit.

Chez une bactérie de type sauvage, l'induction des gènes SOS se feraient selon la séquence : sfiA - lexA - recA - umuC.

Mécanisme d'Action d'un Cancérogène Chimique chez E. coli

Le modèle que nous venons de décrire s'applique en particulier au cas des rayons UV ou de la mitomycine C, des agents qui ont l'ADN pour cible principale.

Le problème est apparemment plus complexe quand on s'adresse à des cancérogènes chimiques qui agissent essentiellement sur les protéines cellulaires. La question qui se pose est de savoir si un produit chimique (ou ses métabolites) peut agir sur des répresseurs cellulaires et déclencher ainsi directement des processus type SOS, ou encore, s'il peut agir comme un mutagène direct.

Pour répondre à cette question, nous avons utilisé l'aflatoxine B1 comme cancérogène modèle (38).

L'aflatoxine B1 est une mycotoxine cancérogène chez l'animal. Elle n'est active chez des bactéries qu'après avoir été métabolisées in vitro par des monoxygénases microsomales. Le métabolite actif produit est probablement l'aflatoxine B1-8,9-oxide. Dans une bactérie, les liaisons covalentes entre l'aflatoxine B1 et l'ADN représentent seulement 2 % des liaisons covalentes totales entre l'aflatoxine et les macromolécules cellulaires.

Nous avons montré (38) que chez des bactéries lysogènes incubées avec de l'aflatoxine B1 activée, le taux de synthèse de la protéine RecA augmente fortement et le répresseur du prophage λ est clivé. Cette dernière réaction exige une synthèse protéique de novo. On peut donc exclure la possibilité d'une action directe des métabolites de l'aflatoxine B1 sur les répresseurs du gène recA ou du prophage λ. Nos résultats suggèrent que les lésions de l'ADN, causées par les métabolites de l'aflatoxine B1,

induisent l'"activation" de la protéine RecA qui peut alors détruire des répresseurs cellulaires spécifiques.

L'activation de la protéine RecA peut rendre compte du fait que des processus épigénétiques (induction lysogénique, filamentation), aussi bien que génétiques (mutagénèse), sont induits dans des bactéries exposées à des cancérogènes chimiques.

Avec un certain nombre d'autres auteurs, nous avons émis l'hypothèse que des "fonctions SOS" pourraient être induites chez les cellules eucaryotes exposées à des cancérogènes chimiques. Suivant cette hypothèse, la transformation cancéreuse résulterait de l'induction de ces fonctions, et non d'une simple accumulation de mutations (36, 47).

Tests à Court Terme

Considérant les données théoriques et expérimentales que nous venons de présenter, nous pouvons maintenant comprendre pourquoi des analogies, mais aussi des différences, existent entre les différents tests bactériens.

Un test de mutagénèse détecte indifféremment les mutagènes "directs" (EMS) et les mutagènes "indirects" (UV, aflatoxine B1). L'effet mutagène peut être révélé par la réversion de mutations bactériennes (comme dans le test développé par le groupe de B. Ames : "Salmonella mutagenicity test") (1) ou à l'aide de systèmes phagiques (36). Par contre, un test d'induction lysogénique, comme l'Inductest (35) ou ses nombreuses variantes (25), détecte uniquement des agents qui induisent les fonctions SOS.

Ces deux types de tests, utilisés conjointement, permettent donc de préciser la nature des lésions de l'ADN. Nous avons pu ainsi établir une distinction entre trois grands types de produits (données expérimen-

tales non montrées et réf. 35-36) :

1) Produits qui ont à la fois une activité inductrice du prophage λ et une activité mutagène. Ces produits qui induisent les fonctions SOS chez les bactéries sont connus pour posséder une activité cancérogène forte chez l'animal (aflatoxine B1, benzo(a)pyrène, 7,12-diméthyl benz(a)anthracène, 2-aminoanthracène, nitrosométhylurée, ...).

2) Produits qui ont une activité mutagène mais pas, ou très peu, d'activité inductrice du prophage λ (EMS, bromure d'éthidium, azoture de sodium, folpet, captan, diméthylnitrosamine). En d'autre termes, ces produits sont essentiellement des mutagènes "directs". Ils n'ont pratiquement pas d'activité cancérogène chez l'animal (ou seulement à des doses très fortes), ce qui n'exclut d'ailleurs pas leur danger à long terme s'ils peuvent agir au niveau des cellules germinales.

Il faut toutefois souligner que les concentrations requises pour détecter l'activité d'un mutagène direct sont généralement très supérieures à celles nécessaires pour induire les fonctions SOS : les concentrations minimales inductrices de mitomycine C ou d'aflatoxine B1 sont respectivement de 0,1 et 10 ng/ml dans l'Inductest, alors que les concentrations minimales mutagènes (chez Salmonella) d'éthylméthanesulfonate ou de diméthylnitrosamine sont supérieures à 100 µg/ml.

3) Produits qui ont une activité inductrice du prophage λ mais pas, ou très peu, d'activité mutagène. Les produits qui appartiennent à cette catégorie possèdent généralement une activité anti-tumorale (bléomycine, néocarzinostatine, 5-fluorouracile, actinomycine D, tallysomycine, mitomycine C). Ces substances causent des lésions qui ne peuvent pas être réparées efficacement même avec l'aide des systèmes de réparation SOS. Toutefois, si aucune lésion létale n'endommage le prophage, celui-ci

s'excise du chromosome bactérien pour donner naissance à des phages révélateurs de l'induction des fonctions SOS.

Une substance chimique qui appartient à ce dernier groupe possède potentiellement une activité antitumorale puisque l'induction des fonctions SOS s'accompagne généralement de la mort de la cellule. Cependant, d'autres exigences devront être remplies pour qu'une telle substance présente des propriétés thérapeutiques intéressantes, en particulier, elle devra posséder une spécificité d'action sur les cellules tumorales (30).

Aspect Quantitatif des Tests Bactériens

En utilisant des tests bactériens, peut-on prédire de façon quantitative l'activité cancérogène, antitumorale, ou mutagène d'un produit chimique chez l'homme ?

Si l'on considère des substances appartenant à une même famille chimique, on est en droit de donner une telle estimation pour un produit par rapport à un autre pris comme référence (2). Cette estimation devient très contestable si l'on considère des produits chimiques appartenant à des familles chimiques différentes. Cela est d'autant plus vrai si l'on utilise comme test des souches bactériennes contenant des plasmides qui augmentent la mutagénèse.

Le plasmide pKM101, qui a été introduit dans certaines souches Salmonella (TA98 et TA100)(1),code de façon constitutive pour des produits qui provoquent une mutagénèse différente de celle (directe ou indirecte) normalement observée chez des bactéries (16, 20). Certaines lésions de l'ADN, qui pourraient être réparées de façon correcte par les enzymes bactériennes, sont prises en charge par les enzymes mutagènes codées par le plasmide. Une substance chimique qui possède une activité mutagène

uniquement chez des bactéries possédant un tel plasmide cause, certes, des dommages à l'ADN, mais ces dommages n'ont généralement aucune conséquence fâcheuse pour une cellule normale. Le risque de "faux positif" (produit actif chez les bactéries mais inactif - non mutagène ou non cancérogène - chez les cellules eucaryotes) est probablement augmenté, et ceci, sans pour autant supprimer le risque de "faux négatif" dû, en particulier, à des problèmes de perméabilité cellulaire ou de métabolisation (1, 2, 7, 36).

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LEGENDES DES FIGURES

Figure 1 : Séquence des événements cellulaires déclenchés par l'endommagement de l'ADN.

Voie A : les bases altérées provoquent des appariements de bases illégitimes pendant la réplication.

Voie B : les lésions résiduelles, dites "non codantes", empêchent la réplication normale de l'ADN et déclenchent les "fonctions SOS".

Dans le cas de mutagènes directs (type éthylméthanesulfonate ou 2-aminopurine) seule la voie métabolique A est utilisée. Au contraire, dans le cas de mutagènes "indirects" (type rayons UV ou métabolites de l'aflatoxine B1), seule la voie B est suivie. Enfin, dans le cas de certains agents alkylants (type méthylméthanesulfonate ou nitrosoguanidine), la voie A est utilisée pour des doses faibles, et la voie B pour des doses fortes.

Figure 2 : Accumulation de la protéine RecA (RecA natif) après endommagement de l'ADN par les rayons ultraviolets.

La courbe hypothétique figurant la production de la forme "activée" de la protéine RecA (RecA protéase) est également représentée.

Figure 3 : La protéine LexA, répresseur des "gènes SOS".

Dans une bactérie en phase exponentielle de croissance, le produit du gène lexA contrôle négativement sa propre synthèse, ainsi que l'expression de plusieurs autres gènes : recA, uvrA (réparation par excision), umuC (réparation mutagène) et sfiA (filamentation cellulaire). Le chromosome est composé de 4×10^6 paires de bases : le prophage λ a une longueur de $4,7 \times 10^4$ paires de bases.

Figure 4 : Modèle d'induction des fonctions SOS

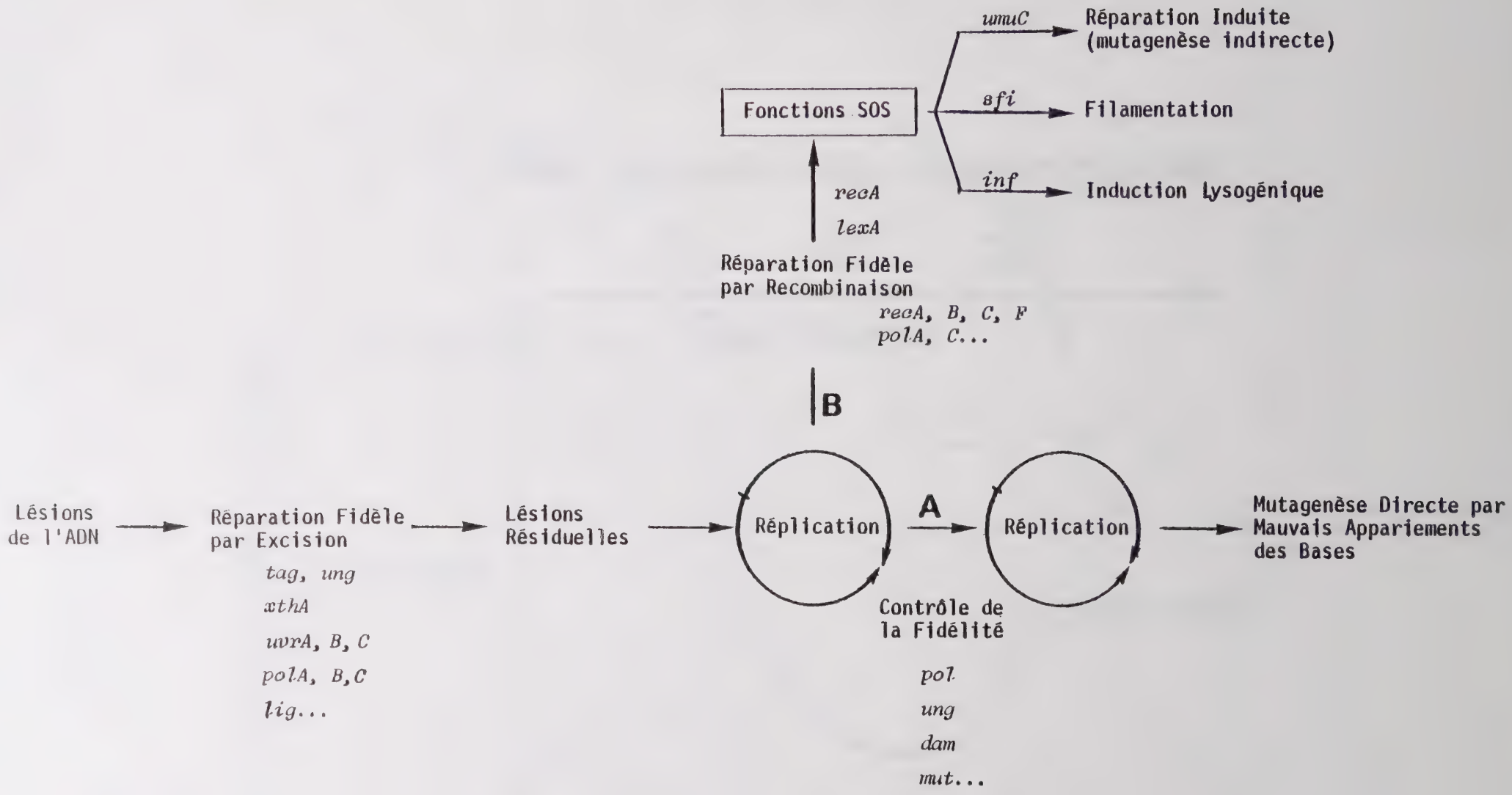

umuC
Réparation Induite
(mutagenèse indirecte)
Fonctions SOS
sfi
Filamentation
inf
Induction Lysogénique
recA
lexA
Réparation Fidèle
par Recombinaison
recA, B, C, F
polA, C...
B
Lésions
de l'ADN
Réparation Fidèle
par Excision
tag, ung
xthA
uvrA, B, C
polA, B,C
lig...
Lésions
Résiduelles
Réplication
A
Réplication
Mutagenèse Directe par
Mauvais Appariements
des Bases
Contrôle de
la Fidélité
pol
ung
dam
mut...

Fig.1

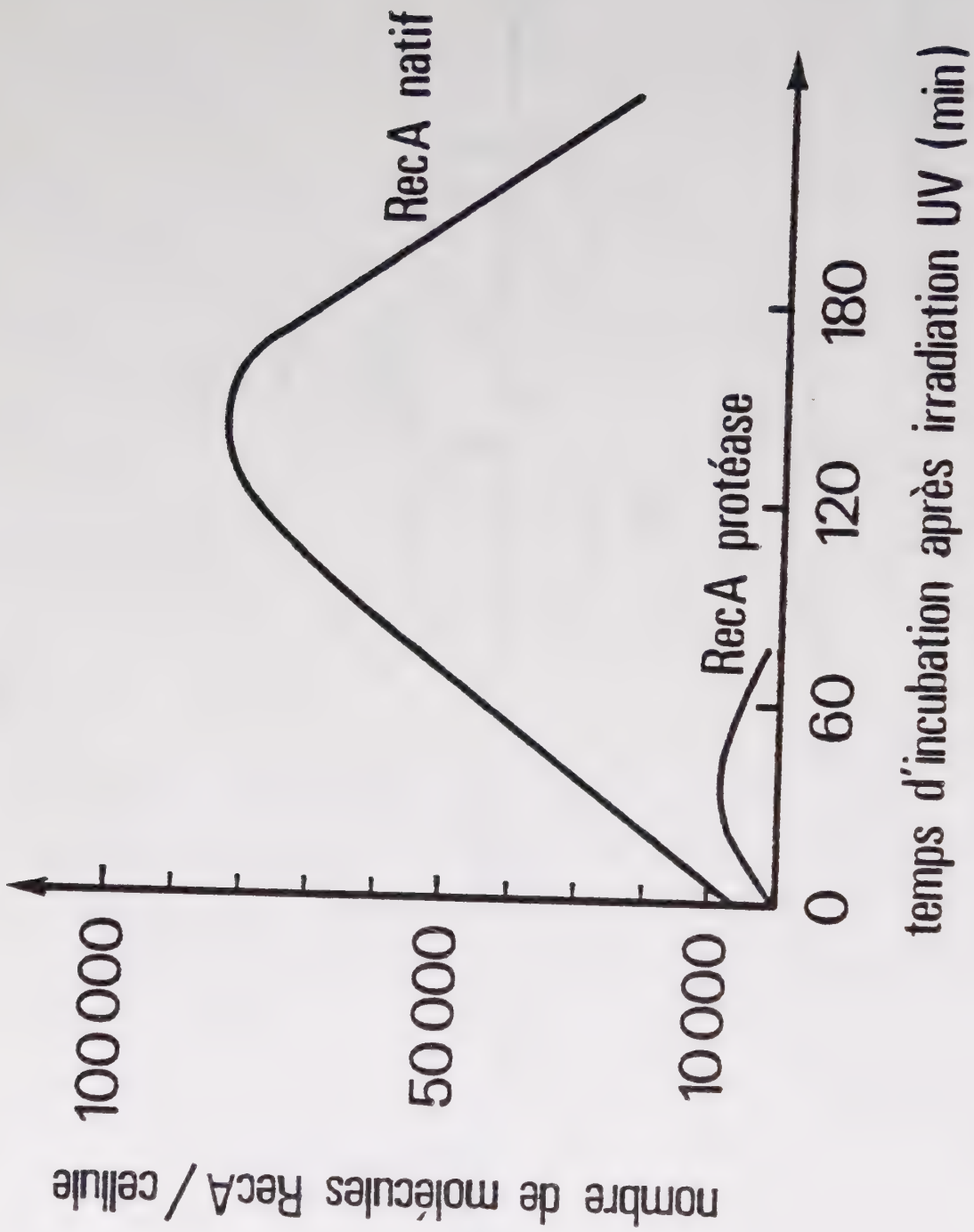

nombre de molécules RecA / cellule
100 000
50 000
10 000
0
60
120
180
temps d'incubation après irradiation UV (min)
RecA natif
RecA protéase

Fig.2

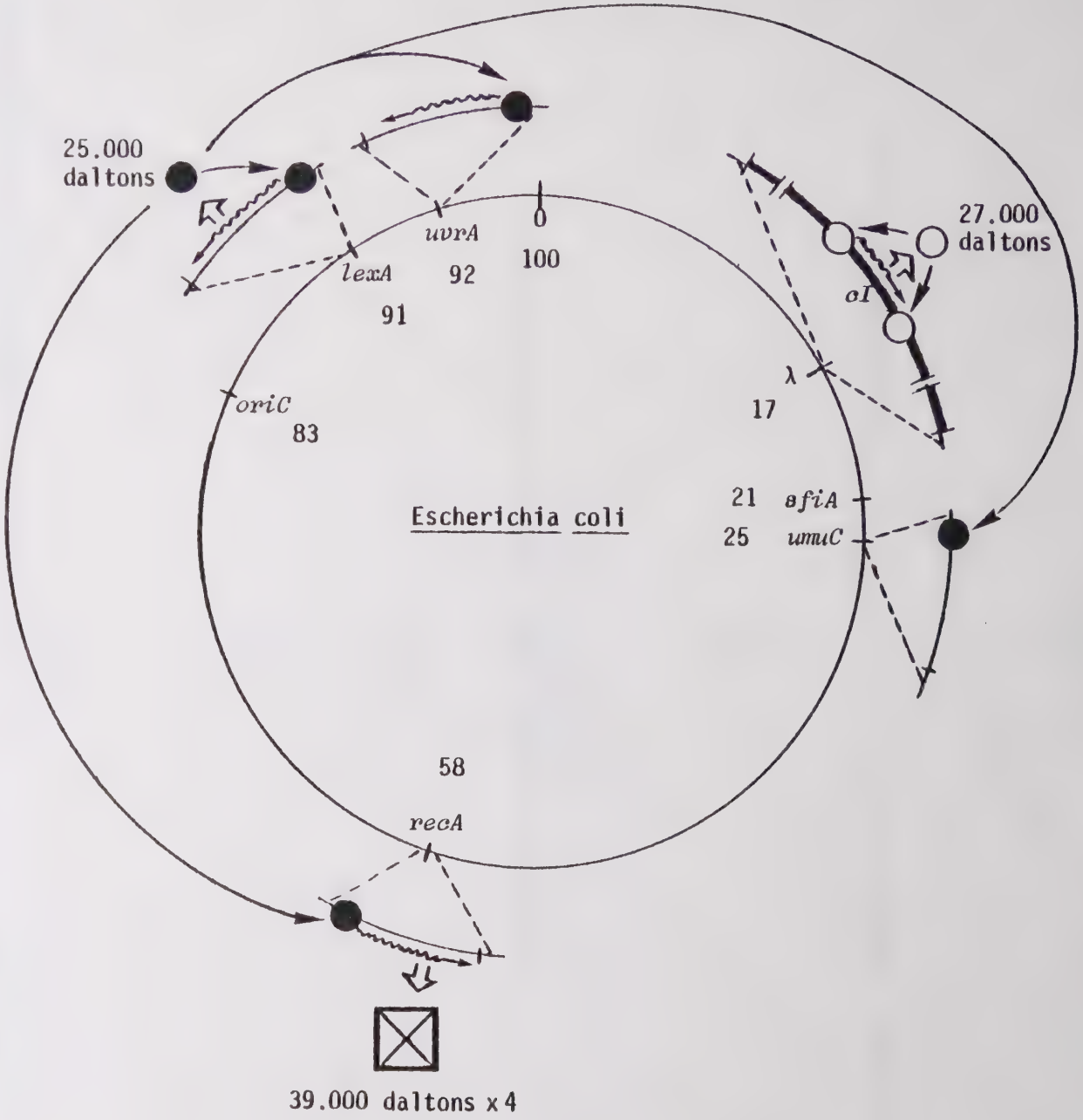
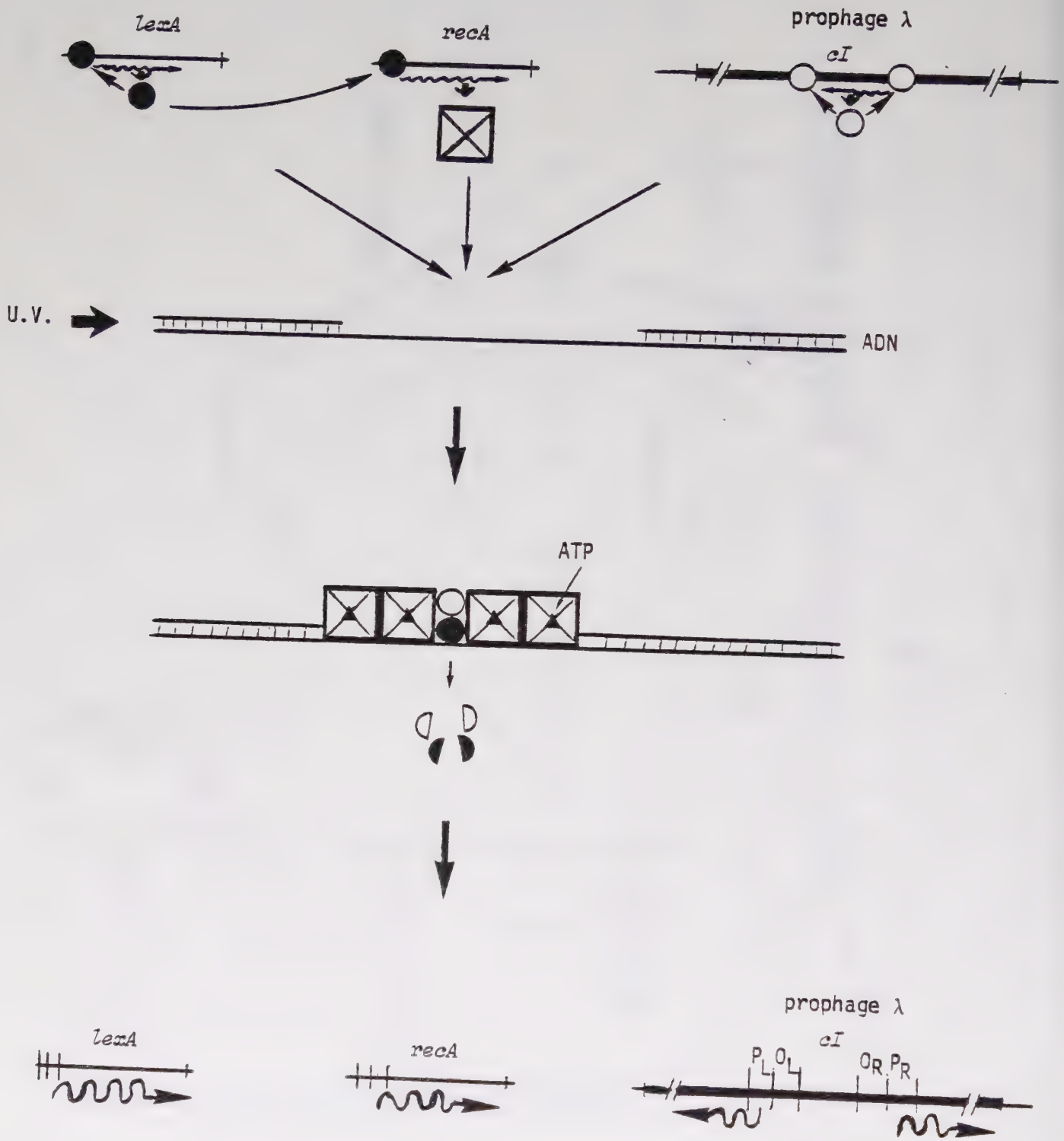

25.000
daltons
uvrA
0
100
lexA
92
91
27.000
daltons
oI
λ
17
oriC
83
Escherichia coli
21
sfiA
25
umuC
58
recA
39.000 daltons x 4

Fig.3


lexA
recA
prophage λ
cI
U.V.
ADN
ATP
lexA
recA
prophage λ
cI
P_L O_L
O_R P_R

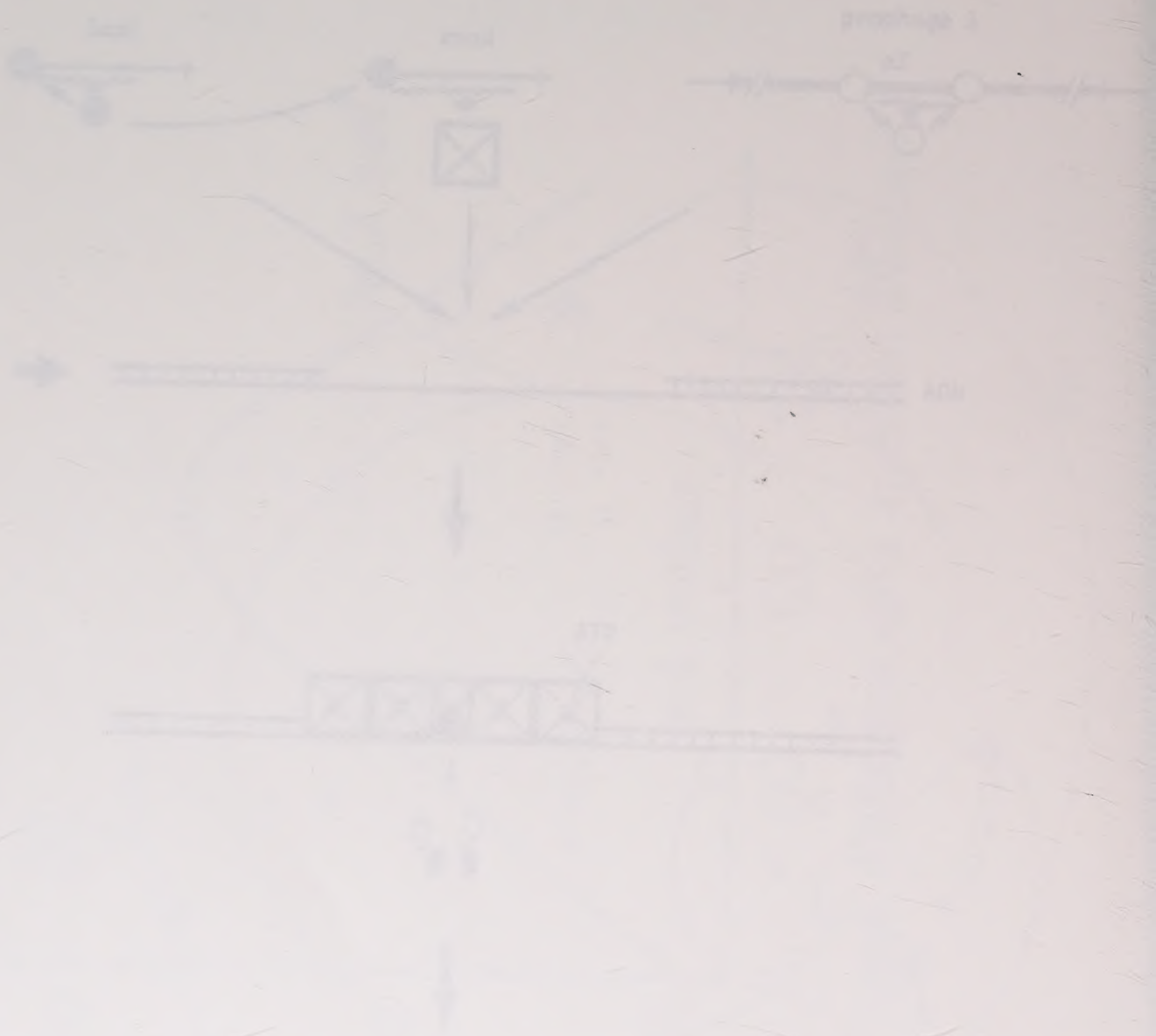



Fig.4